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DOCKET NO.: 201859US0PCT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF :

JACQUES THEZE, ET AL. : EXAMINER: MERTZ, P.

SERIAL NO: 09/720,828 : GROUP ART UNIT: 1646

FILED: MARCH 1, 2001 : ALLOWED: SEPTEMBER 11, 2003

FOR: PEPTIDES OF IL-2 AND DERIVATIVES THEREOF AND THEIR USE AS THERAPEUTIC AGENTS

PETITION UNDER 37 C.F.R. §1.181(a)(3)

COMMISSIONER FOR PATENTS
Alexandria, VA 22313-1450

SIR:

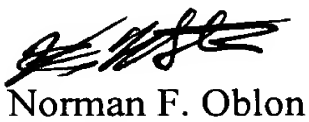
Petitioners respectfully request the Office to invoke the supervising authority of the Commissioner and consider Petitioner's Comments on Statement of Reasons for Allowance, concurrently filed herewith, and also indicate that the references cited on the International Search Report filed on January 16, 2001, has been considered. Petitioners respectfully request that the Examiner acknowledge consideration of same by providing Applicants with an *initialed* copy of Form PTO-1449 enclosed herewith listing these references. Petitioners submit herewith a copy of the International Search Report, as filed, along with a copy of the references to be considered and the date-stamped filing receipt evidencing the timely filing thereof.

In addition, Petitioners request the Office to provide acknowledgment of the claim to priority. A copy of the Request for Priority Acknowledgment under 35 U.S.C. 119 and the International Convention is enclosed herewith, as concurrently filed with the Comments on Statement for Reasons for Allowance, for the Examiner's convenience.

For the reasons given above, this petition should be GRANTED.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.



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O.S.&M. File No. 201859US0PCT By NFO/dpp FF Due Date 01-16-01

Serial No. New U.S. PCT Application based on PCT/IB99/01424

In the Matter of the Application of Jacques THEZE, et al.

For PEPTIDES OF IL-2 AND DERIVATIVES THEREOF AND THEIR USE AS THERAPEUTIC AGENTS

The following has been received in the U.S. Patent Office on the date stamped hereon:

[ ] _ pgs. Specification __ Claims (English Translation) __ Sequence Listing

[ ] Combined Declaration, Petition & Power of Attorney (_ pages)

[ ] Petition of Revive Under 37 C.F.R. 1.137 (b)

[➢] Notice of Priority;

[➢] Check for $ 1,160.00 ___; [➢] Dep. Acct. Order Form

[ ] Assignment _____ pages/PTO-1595

[ ] Drawings __ sheets

[➢] PCT Transmittal Letter

[ ] Preliminary Amendment

[ ] PCT/IB/304

[ ] PCT/IB/308

[ ] International Preliminary Examination Report

[ ] Information Disclosure Statement; [ ] PTO-1449

[ ] Cited References ( )

[ ] Statement of Relevancy

[ ] List of Related Cases [ ] Cited Pending Applications (#)

[➢] International Search Report

[➢] Request for Consideration of Documents Cited in International Search Report

[ ] Letter Regarding Translation of Annexes

[ ] Translation of Annexes to International Preliminary Examination Report

SERIAL NO. 09/720828

DATE RECEIVED

[ ] CLAIMING SMALL ENTITY STATUS

Image

1646

OBLON
SPIVAK
McCLELLAND
MAIER
&
NEUSTADT
P.C.

ATTORNEYS AT LAW

Docket No.: 201859US0PCT

COMMISSIONER FOR PATENTS
ALEXANDRIA, VIRGINIA 22313

RE: Application Serial No.: 09/720,828
Applicants: Jacques THEZE, et al.
Filing Date: March 1, 2001
For: PEPTIDES OF IL-2 AND DERIVATIVES THEREOF AND THEIR USE AS THERAPEUTIC AGENTS
Group Art Unit: 1646
Examiner: Mertz, Prema M.
Allowed: September 11, 2003

SIR:

Attached hereto for filing are the following papers:

Petition Under 37 C.F.R. §1.181(a)(3)
Copy of Date-stamped Filing Receipt dated January 16, 2001
Copy of Request for Priority filed January 16, 2001
Copy of International Search Report filed January 16, 2001
Copy of Cited References (5) filed January 16, 2001

Our check in the amount of **$0.00** is attached covering any required fees. In the event any variance exists between the amount enclosed and the Patent Office charges for filing the above-noted documents, including any fees required under 37 C.F.R 1.136 for any necessary Extension of Time to make the filing of the attached documents timely, please charge or credit the difference to our Deposit Account No. 15-0030. Further, if these papers are not considered timely filed, then a petition is hereby made under 37 C.F.R. 1.136 for the necessary extension of time. A duplicate copy of this sheet is enclosed.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
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Docket No.: 201859US0PCT

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NEUSTADT
P.C.
ATTORNEYS AT LAW

COMMISSIONER FOR PATENTS
ALEXANDRIA, VIRGINIA 22313

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DOCKET NO.: 201859US0PCT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF: Jacques THEZE, et al.

SERIAL NO.: NEW U.S. PCT APPLICATION

FILED: HEREWITH

INTERNATIONAL APPLICATION NO.: PCT/IB99/01424

INTERNATIONAL FILING DATE: 16 July 1999

FOR: PEPTIDES OF IL-2 AND DERIVATIVES THEREOF AND THEIR USE AS THERAPEUTIC AGENTS

REQUEST FOR PRIORITY UNDER 35 U.S.C. 120 AND THE INTERNATIONAL CONVENTION

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In the matter of the above-identified application for patent, notice is hereby given that the applicant claims as priority:

COUNTRY	APPLICATION NO.	DAY/MONTH/YEAR
UNITED STATES	09/116,594	16 July 1998

A certified copy of the corresponding Convention application(s) was submitted to the International Bureau in PCT Application No. **PCT/IB99/01424.**

Respectfully submitted,
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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/IB 99/01424

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K16/24 C07K14/55 C12N15/26 G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ECKENBERG R ET AL., : "Analysis of human IL-2/IL-2 receptor beta chain interactions: Monoclonal antibody H2-8 and new IL-2 mutants define the critical role of alpha helix-A of IL-2." CYTOKINE , vol. 9 (7), 1997, page 488-498 XP000856633 cited in the application the whole document --- -/--	1-4,6-8, 16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 December 1999	14/12/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mateo Rosell, A.M.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter: nal Application No
PCT/IB 99/01424

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	MOREAU J-L ET AL., : "Characterization of a monoclonal antibody directed against the NH2 terminal area of interleukin-2 (IL-2) and inhibiting specifically the binding of IL-2 to IL-2 receptor beta chain (IL-2R-beta)" MOLECULAR IMMUNOLOGY, vol. 32 (14-15), 1995, page 1047-1056 XP000856750 cited in the application the whole document ---	1-8
A	XU D ET AL., : "Biological and receptor-binding activities of human interleukin-2 mutated at residues 20Asp, 125Cys or 127Ser." EUROPEAN CYTOKINE NETWORK , vol. 6 (4), 1995, page 237-244 XP000856616 the whole document ---	6-8, 16-24
X	WO 91 02000 A (SERAGEN INC) 21 February 1991 (1991-02-21) page 9 abstract ---	9,13,16
X	WO 90 00565 A (AMGEN INC) 25 January 1990 (1990-01-25) page 25 page 4, line 1 -page 5, line 20 -----	9,13,16

ANALYSIS OF HUMAN IL-2/IL-2 RECEPTOR β CHAIN INTERACTIONS: MONOCLONAL ANTIBODY H2-8 AND NEW IL-2 MUTANTS DEFINE THE CRITICAL ROLE OF α HELIX-A OF IL-2

Ralph Eckenberg,[1] Di Xu,[2] Jean-Louis Moreau,[1] Marc Bossus,[3] Jean-Claude Mazié,[4] André Tartar,[1] Xin Yuan Liu,[5] Pedro M. Alzari,[6] Jacques Bertoglio,[2] Jacques Thèze[1]

Interleukin 2 (IL-2) interacts with a receptor (IL-2R) composed of three subunits (IL-2Rα, IL-2Rβ and IL-2Rγ). IL-2Rβ plays a critical role in signal transduction. An anti-human IL-2 mAb (H2-8) produced after immunization with peptide 1-30 of IL-2 was found to recognize the region occupied by Asp20, at the exposed interface between α-helices A and C. Muteins at position 17 and 20 are not recognized by mAb H2-8. mAb H2-8 specifically inhibits the IL-2 proliferation of TS1β cells which are dependent on the expression of human IL-2Rβ chain for IL-2 proliferation. Substitution at internal position Leu17 demonstrates that this position is essential for IL-2 binding and IL-2 bioactivity. New IL-2 mutants at position Asp20 have been analysed. Substitutions Asp → Asn, Asp → Lys, Asp → Leu, show a correlation between diminished affinity for IL-2 receptor and reduced bioactivity measured on TS1β cells. Mutein Asp → Arg lose affinity for IL-2R and bioactivity simultaneously. Furthermore, during the course of the study we have found that mutein Asp20 → Leu is an IL-2 antagonist. The biological effects of mAb H2-8 and the properties of new mutants at positions 17 and 20 demonstrate that this region of α helix-A is involved in IL-2–IL-2Rβ interactions.

The effects of human interleukin 2 (IL-2) on its target cells are mediated through specific cell surface receptors (IL-2R).[1-4] IL-2R comprises at least three subunits encoded by different genes.[5,6] The first component to be identified, IL-2Rα, is a 55-kDa protein that binds IL-2 with a K_d of $\simeq 10$ nM.[7,8] The role of IL-2Rα[9] and the influence of IL-2 on IL-2Rα gene expression have been studied in our laboratory.[10,11] The second IL-2R component, IL-2Rβ, is a 75-kDa protein with a large intracytoplasmic domain (286 aa).[12-14] The last component to be identified, IL-2Rγ, is a 64-kDa protein.[15,16] IL-2Rβ and IL-2Rγ belong to the haematopoietin receptor family, whereas IL-2Rα belongs to another family of molecules.[17] In the mouse system all three chains are required to form a functional receptor.[18,19] In the human system two receptors are functional. When associated, human IL-2Rβ plus IL-2Rγ form an intermediate affinity receptor with a K_d of $\simeq 1$ nM, whereas expression of all three chains leads to the formation of a high affinity IL-2R ($K_d \simeq 10$ pM).

The structure of IL-2[20] is composed of a compact core bundle of four antiparallel α helices connected by three loops (Fig. 1). Some of the interactions between IL-2 and IL-2Rα[21,22] and IL-2Rγ subunits[23,24] have been elucidated, but less is known concerning IL-2–IL-2Rβ interaction, despite the fact that IL-2Rβ chain plays a very critical role in signal transduction.[25]

It has been initially shown that one substitution Asp20 by Lys (mutant D20K) prevents binding to IL-2Rβ.[26] In a recent report the role of the sequence (Leu17, Leu18, Leu19, Asp20, Leu21) from IL-2 α helix A, in IL-2–IL-2Rβ interactions was analysed by cassette mutagenesis.[27] However, the data were difficult to interpret since most of the proteins produced have multiple mutations inside and outside of the sequence of interest. Only one analogue with a single mutation was studied (L21V). More surprisingly it was reported

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Received 12 September 1996; accepted for publication 2 December 1996

1043–4666/97/070488 + 11 $25.00/0/ck960192

KEY WORDS: IL-2 structure/function/IL-2 receptors/IL-2 muteins

in this study that deletion of the segment spanning residues 17–31 (Del1) gives a protein with full agonist activity.

To clarify this critical question we have used two complementary approaches. The immunochemical properties of a mAb (H2-8) directed against a peptide consisting of IL-2 residues 1–30 were characterized. Three new IL-2 muteins with point mutations at position 20 were analysed. The role of Leu17 (other Leu residue near Asp20 in the sequence and involved in core packing) was also studied. Altogether the results firmly establish the role of α helix A of IL-2 in IL-2–IL-2Rβ interactions and are discussed in the context of a structural model of the IL-2–IL-2R complex.[28]

RESULTS

mAb H2-8 binds to α helix A sequences of IL-2

The inhibitory effect of purified mAb H2-8 was first assayed on the binding of ^{125}I-labelled IL-2. Its effects were measured on two transfectants derived from a mouse cell line expressing only mIL-2Rγ. Transfectant TS1β and TS1α were obtained after transfection with human IL-2Rβ and human IL-2Rα cDNA, respectively. Both cell lines bind IL-2. mAb H2-8 inhibits the binding of IL-2 to TS1β without significantly affecting IL-2 binding to TS1α (data not shown).

The binding properties of mAb H2-8 were studied by enzyme-linked immunosorbent assay (ELISA). Plates were coated with either IL-2 or peptides 1–22 or 1–30. mAb H2-8 binds IL-2 and peptide 1–30 but does not recognize peptide 1–22. As control mAb 19B11 (previously characterized) recognized both peptides (Fig. 2).

Binding inhibition experiments were performed to characterize further the specificity of mAb H2-8. Plates were coated with IL-2 and a concentration of mAb H2-8 giving approximatively 50% of maximum binding was used. H2-8 was preincubated with different peptides including five decapeptides (1–10, 5–15, 10–20, 15–25, 20–30). Under our experimental conditions only IL-2 and peptide 1–30 were able to inhibit the binding of mAb H2-8 to IL-2. Peptide 1–30 was the most efficient inhibitor in these experiments (Fig. 2). This result is compatible with the fact that isolated peptide 1–30 folds in an α helical configuration (α-helix content of 50% ± 7%), whereas peptide 1–22 does not (0% ± 5%) as measured by circular dichroism. Therefore peptide 1–30 may adopt some unique structural conformation very close to that of native IL-2. As control the binding of mAb 19B11 is inhibited not only by IL-2 but also by peptides 1–10, 1–22 and 1–30. This confirms that the epitope of mAb 19B11 is near

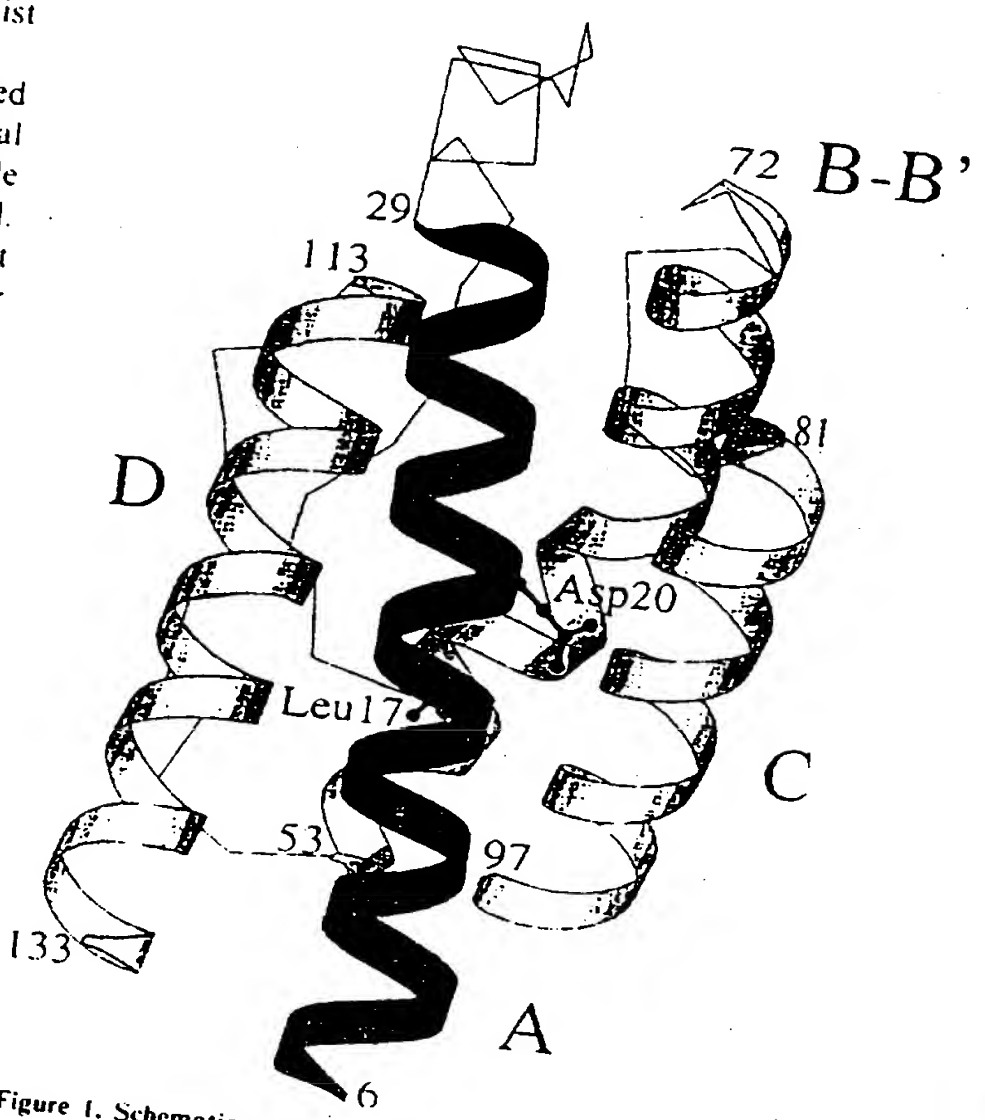


Figure 1. Schematic representation of the human IL-2 structure.

The protein contains 133 amino acid residues (molecular weight 15–18 kDa, depending on the degree of glycosylation). Four α-helices, denoted A (residue positions 6–29), B-B' (positions 53–72), C (positions 81–97), and D (positions 113–133) surround a central hydrophobic core. Residues Leu17 and Asp20 (see text) occur in the N-terminal helix A. The structure of the loop between α-helix C and α-helix D was undetermined. Atomic coordinates were obtained from the Brookhaven Protein Data Bank[31] entry 3ink, deposited by D. B. McKay.[29] The figure was drawn with the program Molscript.[44]

the NH_2 terminal position of IL-2 as previously suggested.[29]

Involvement of aa at positions 17 and 20 in mAb H2-8 recognition

The reactivity of mAb H2-8 to various IL-2 mutants including one mutant at position 17 (Leu → Asp), four mutants at position 20 (Asp → Asn; Asp → Lys; Asp → Arg and Asp → Leu) and a double mutant 17-20 (Leu17 → Asp and Asp20 → Leu) were tested by Western blot analysis (Fig. 3).

Figure 3 shows that mAb H2-8 does not recognize mutations at position 20 or the double mutant 17-20. Recognition of the mutation at position 17 is also affected. As positive control the results obtained with mAb 2C4 that recognize an epitope near the NH_2 terminal area of IL-2 are shown. Since this mAb (as 19B11) recognizes peptide 1–10 which bears no

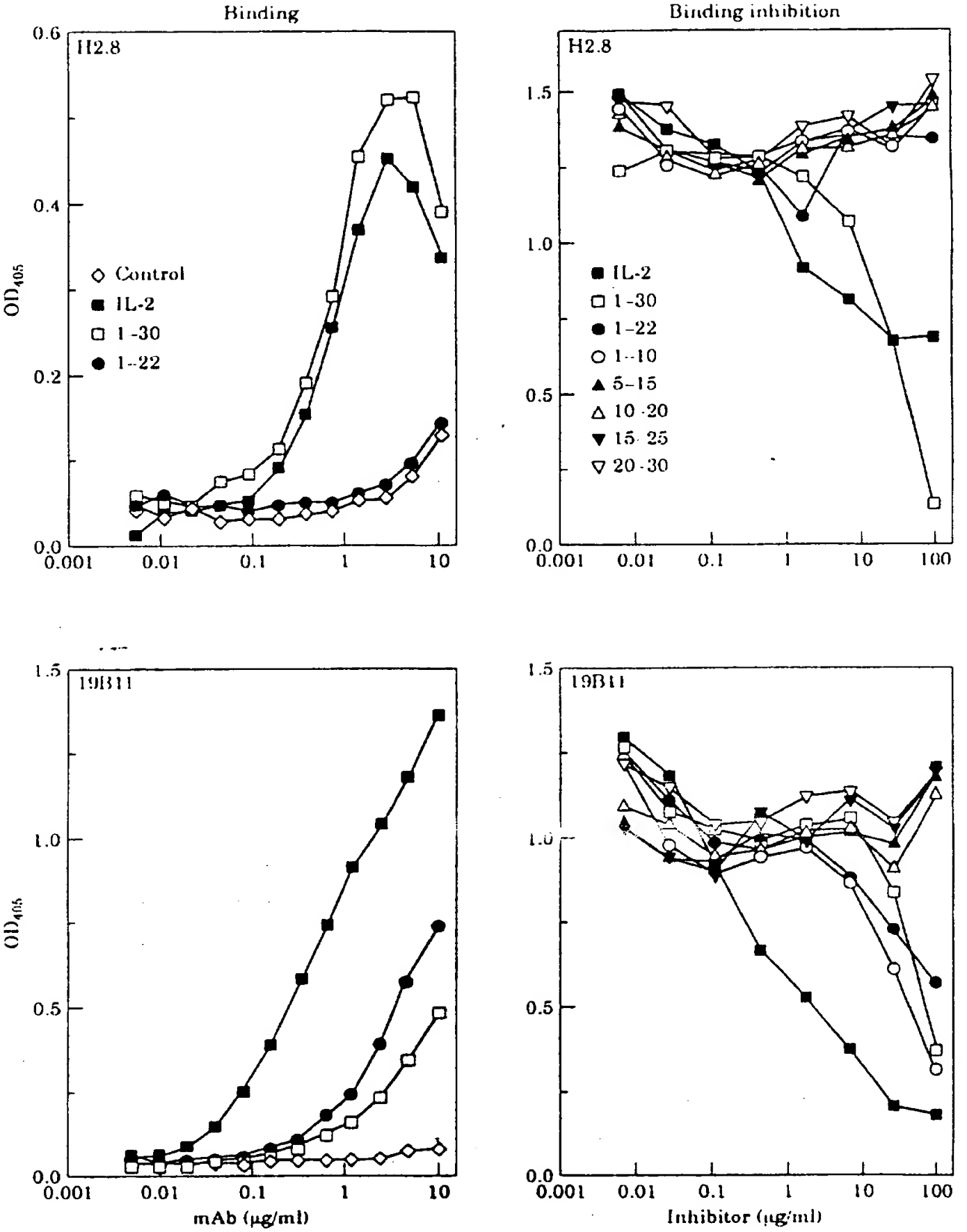


Figure 2. Binding and binding inhibition of mAb H2-8 and 19B11.

Binding experiments: plates were coated with IL-2, peptide 1–22 or peptide 1–30. Control is represented by non-coated plates. Binding of mAb was revealed with alkaline phosphatase goat anti-mouse polyvalent Ig conjugate.

Inhibition experiments: concentrations of mAbs H2-8 or 19B11 giving half maximal binding on IL-2-coated plates were used. These dilutions were mixed for 1 h at 37 C with the indicated concentration of inhibitors before addition to wells coated by IL-2.

mutation, its binding to IL-2 is not affected (Fig. 3). Similarly, mutations at position 125 and/or 127 do not affect binding to mAbs H2-8 and 2C4, and serve as additional controls (Fig. 3). ELISA experiments performed with all the mutants support the data obtained with Western blots (data not shown).

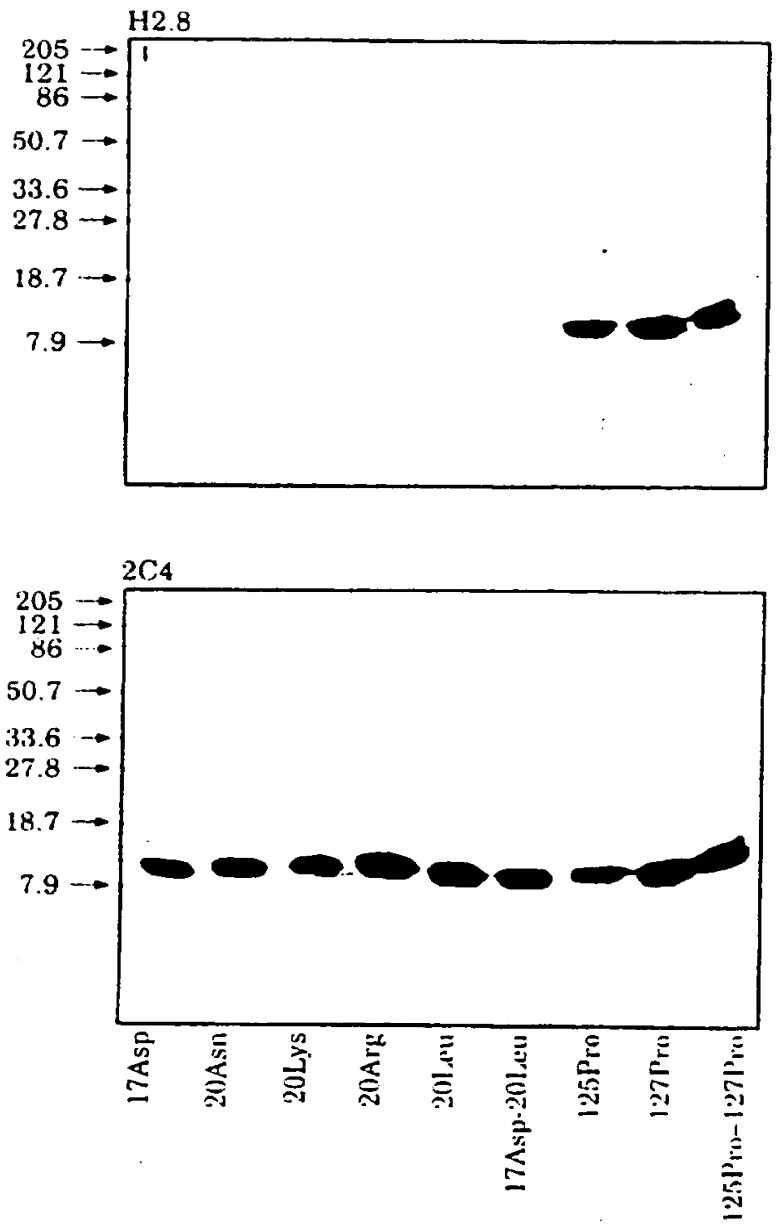


Figure 3. Detection of IL-2 mutants by mAbs H2-8 and 2C4-Western blot.

IL-2 mutants were separated in SDS-PAGE and electroblotted onto nitrocellulose. After saturation the membrane was incubated with the indicated mAb followed by peroxidase-conjugated goat anti-mouse Ig. The blots were developed using enhanced chemiluminiscent detection system.

mAb H2-8 and 19B11 have similar properties: both bind to the NH_2 terminal end of IL-2 and specifically inhibit the binding of IL-2 to IL-2Rβ chain. Since both antibodies recognize sequences located in peptide 1–30 it was of interest to compare the relationship between the corresponding epitopes. Plates coated with mAb H2-8 were used to bind peptide 1–30. The binding of mAb 19B11 to these plates was positive, thus indicating that the epitopes of mAbs H2-8 and 19B11 do not overlap significantly. Various controls performed to verify these results are shown (Fig. 4). The binding of 19B11 is strictly dependent on the presence of peptide 1–30 and on the coating by mAb H2-8. Results obtained with mAb 3H9 recognizing the peptide 30–54 further demonstrated the specificity of the data presented in Figure 4.

Biological properties of mAb H2-8

The biological properties of mAb H2-8 were evaluated on the proliferation of the IL-2- or IL-9-dependent TS1β cell line (Fig. 5). In these experiments the IL-2 mutant IL-2 Pro125 (Cys → Pro) was used. This mutation slightly reduces the affinity of IL-2 for IL-2R (Table 1) without affecting the maximum proliferation obtained when higher concentrations of mutant are used (Fig. 6).

Figure 5 shows that different concentrations of mAb H2-8 reduce the IL-2 proliferation of TS1β. A progressive shift of the IL-2 titration curve is observed with increasing concentrations of mAb H2-8. Inhibitory effects of mAb H2-8 are comparable to those obtained with mAb 19B11, which was also

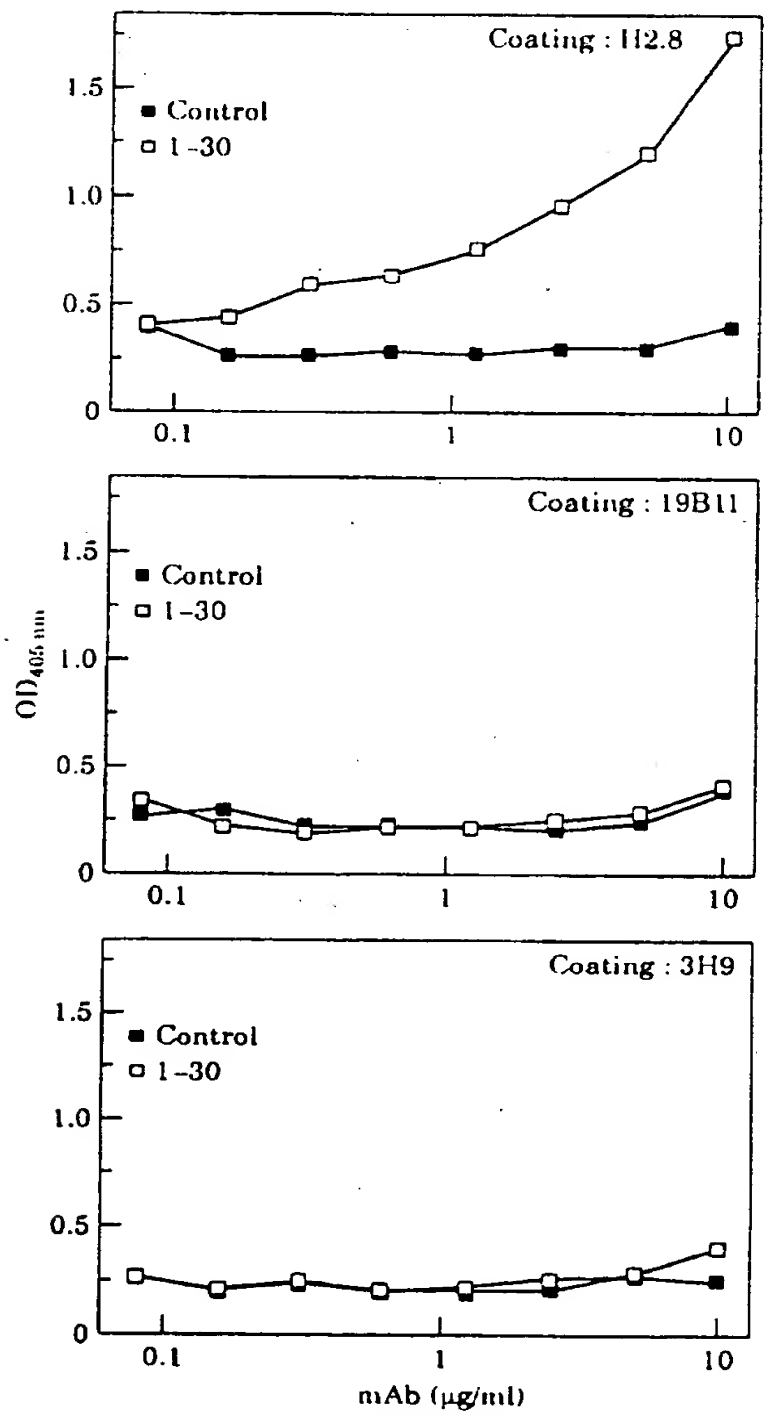


Figure 4. Binding of mAbs H2-8, 19B11 and 3H9 on peptide 1–30.

Plates were coated with mAbs H2-8, 19B11 or 3H9, and were incubated with peptide 1–30 as described. Binding of biotine labelled mAb 19B11 was assayed by alkaline phosphatase–avidin conjugate.

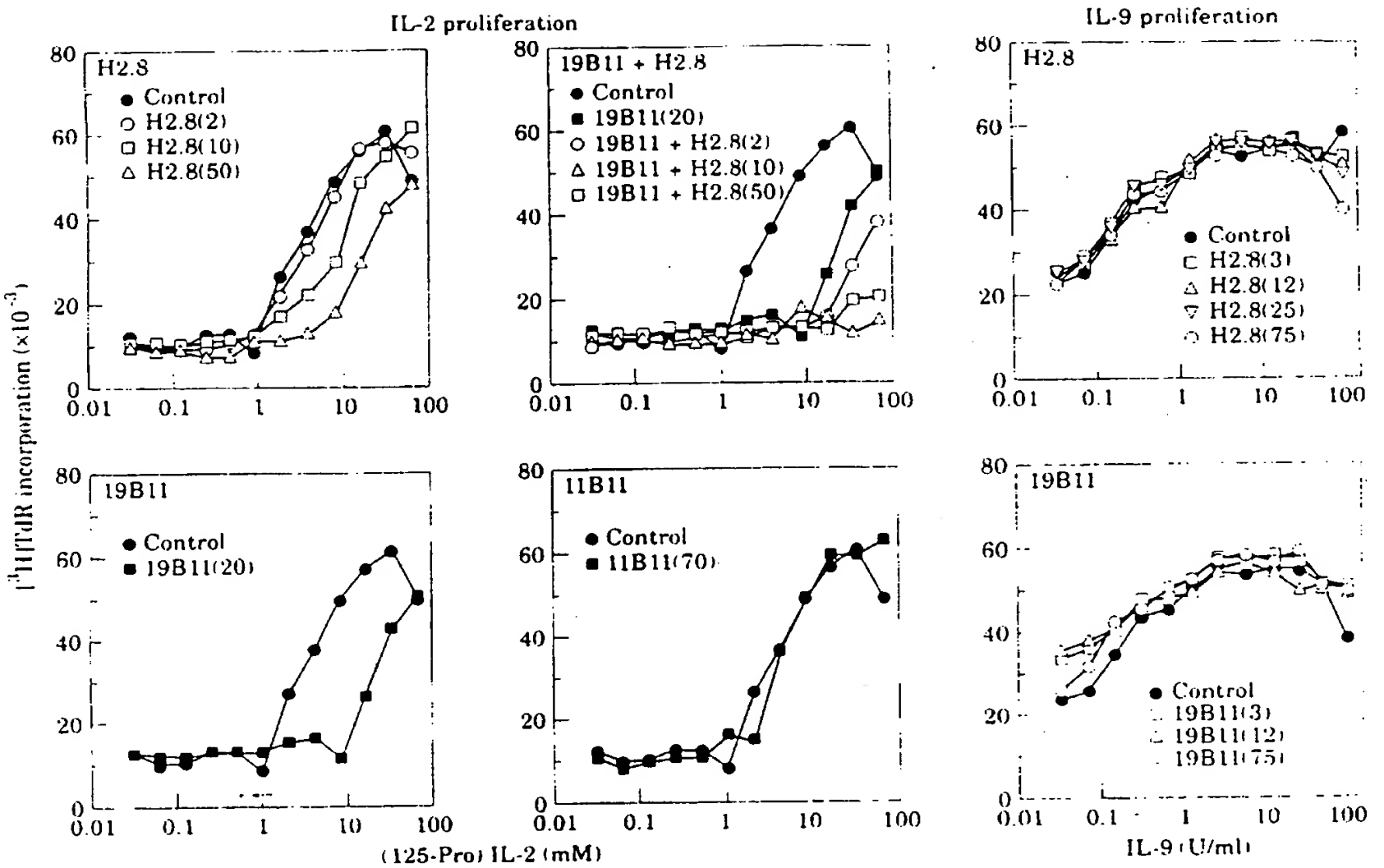


Figure 5. Biological effects of mAb H2-8 on IL-2 Pro125.

Different concentrations of IL-2 Pro125 were tested on the proliferation of TS1β cells (IL-2Rα⁻, human IL-2Rβ⁺, mouse IL-2Rγ⁺). The proliferation was measured by [³H]TdR incorporation as indicated.

Various concentrations (indicated in parenthesis—μg/ml) of mAb H2-8, 19B11 or mixtures of H2-8 + 19B11 were tested and the effects measured on the reduction of [³H]TdR incorporation. As control the absence of effects of mAb H2-8 and 19B11 on IL-9 dependent proliferation of TS1β cells was verified. The absence of inhibition of the IL-2 Pro125 proliferation was also verified in the presence of mAb 11B11 (anti-mouse IL-4).

found to inhibit the proliferation of cells bearing high affinity IL-2R.[39] Addition of both mAb H2-8 and 19B11 completely abolishes IL-2 proliferation even at a very high dose of IL-2.

As controls Figure 5 shows that mAb 11B11 (specific for mouse IL-4) does not affect the IL-2-dependent proliferation of TS1β. IL-9-induced proliferation of TS1β is also not affected by either H2-8 or 19B11.

Binding of IL-2 muteins to IL-2 receptors

To determine the binding capacity of IL-2 muteins to IL-2R, we measured the competitive displacement of ^{125}I-labelled IL-2 to different cell lines expressing

TABLE 1. Competitive IL-2R binding of IL-2 muteins on CTLL-2, YT and TS1β cell lines

	CTLL-2*		YT*		TS1β*	
Cell	IC_{50}	(%WT)†	IC_{50}	(%WT)†	IC_{50}	(%WT)†
Wild-type	2.8×10^{-10}	100	5.7×10^{-9}	100	6×10^{-8}	100
17 ASP	2.8×10^{-8}	1	4.1×10^{-7}	1.3	2×10^{-6}	3.0
20 ASN	3.9×10^{-8}	0.7	2.9×10^{-8}	19.6	4×10^{-7}	15
20 LYS	1.5×10^{-7}	0.2	2.2×10^{-7}	2.5	7×10^{-7}	8.5
20 LEU	8.6×10^{-8}	0.3	4.5×10^{-7}	1.3	8×10^{-7}	7.5
20 ARG	—‡		—‡		—	
125 PRO	1.7×10^{-9}	16	2.8×10^{-8}	20.4	1.5×10^{-7}	40
127 PRO	1.4×10^{-8}	2	5.4×10^{-8}	10.7	ND§	
125/127 PRO	2.8×10^{-8}	1	3.4×10^{-8}	16.9	ND§	

*Concentration of ^{125}I-labelled IL-2 used for binding: CTLL-2 = 200 pM; YT = 1 nM and TS1β = 3 nM.
†%WT = [(IC_{50}) wild-type/(IC_{50}) mutant] × 100.
‡— = not detectable ([IC_{50}] > 10^{-6} M IL-2).
§ND = not determined.

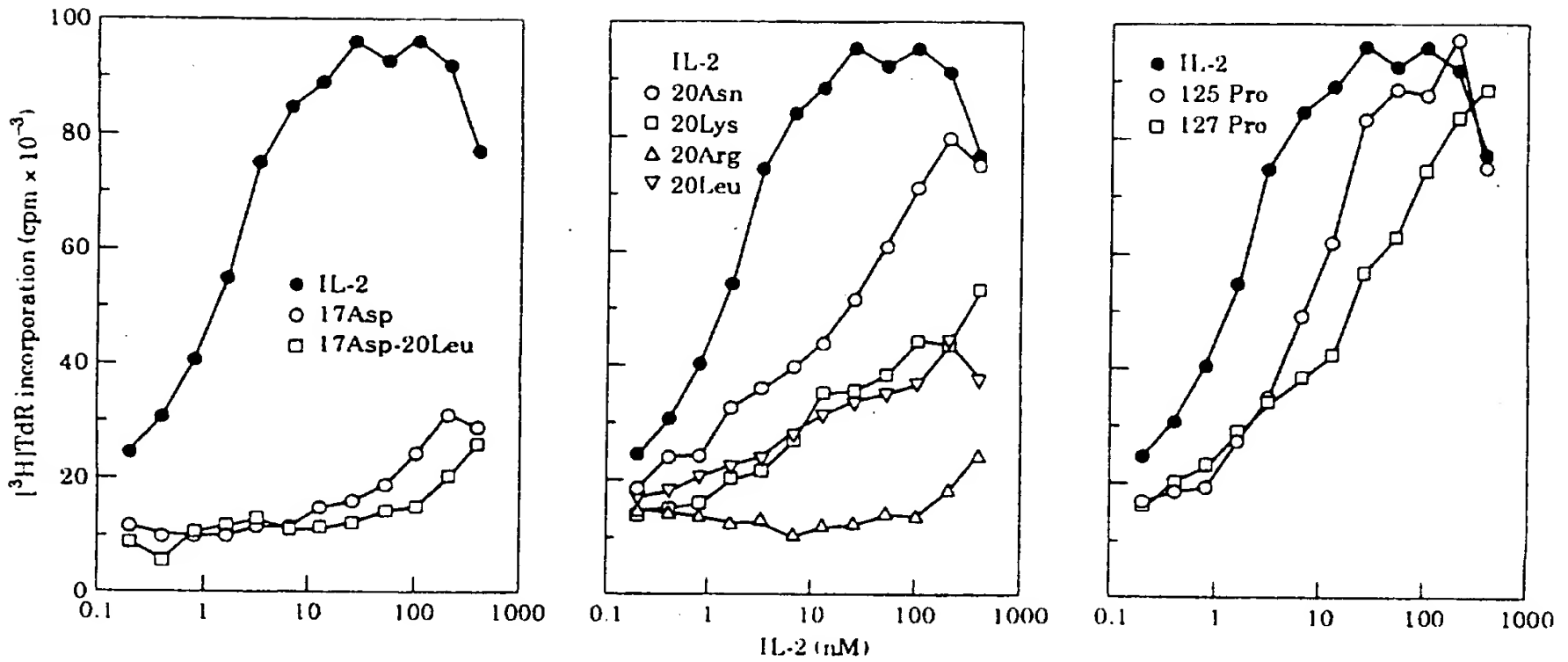


Figure 6. Biological activity of IL-2 mutants tested on TS1b cells.

Various concentrations of the different muteins were tested on the proliferation of TS1β cells. Proliferation was measured by [³H]TdR incorporation as described

Background proliferation in the absence of cytokine was not substracted (10–20 × 10^3 cpm).

different chains of the IL-2R. CTLL-2 express mouse IL-2Rα, β and γ and the high-affinity IL-2R. YT express human IL-2Rβ and IL-2Rγ and the intermediate affinity IL-2R. TS1β express the hetero-specific IL-2R made of human IL-2Rβ and mouse IL-2Rγ. The results are presented Table 1. Mutation at position 17 (Leu → Asp) and mutations at position 20 (Asp → Lys and Asp → Leu) strongly reduce the affinity of IL-2 for the three type of receptors. Mutation at position 20 (Asp → Arg) abolishes the capacity of IL-2 to bind to the different IL-2R. Surprisingly substitution at position 20 (Asp → Asn) reduces the capacity to bind to both human intermediate and mouse high-affinity IL-2R. One can also see in Table 1 that mutation at position 125 (Cys → Pro) reduces the affinity of IL-2 to the three types of IL-2R studied.

Biological properties of IL-2 muteins with substitutions at positions 17 and 20

To investigate further the role of amino acids at positions 17 and 20 it was of interest to measure the biological properties of the corresponding mutants on the proliferation of TS1β cells (Fig. 6). Mutation (Leu17 → Asp) strongly reduces the IL-2 bioactivity. Mutation (Asp20 → Arg) completely abolishes the biological activity of IL-2 in agreement with the lack of IL-2R binding (Table 1). Mutation (Asp20 → Lys) and (Asp20 → Leu) strongly reduce the bioactivity. Mutation (Asp20 → Asn) diminishes the activity but high amounts of the corresponding mutein completely restore the maximum proliferation of TS1β cells. Mutations at position 125 (Cys125 → Pro) or 127 (Ser127 → Pro) which break the α helical structure by introducing a proline residue into helix D were used as controls in these experiments.

Mutein IL-2 (Asp20 → Leu) which binds to IL-2R (Table 1) also exhibits an antagonist effect on IL-2 proliferation (Fig. 7). When IL-2 titration curves were performed in the presence of increasing amounts of the Leu20 mutein, the IL-2 response of TS1β cells was progressively reduced. This effect is specific since the IL-9-dependent proliferation of TS1β is not affected by mutein 20 Leu (Fig. 7).

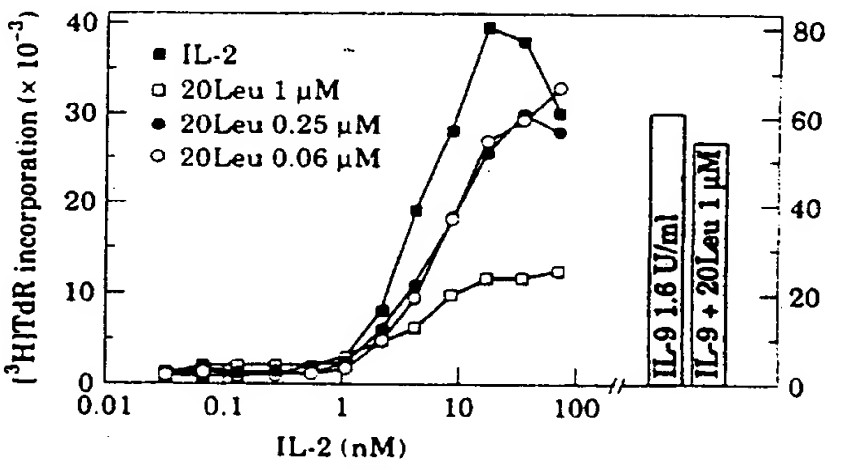


Figure 7. Inhibition of IL-2 proliferation by IL-2 mutant Leu20.

IL-2 proliferation was tested on TS1β cells as described in Materials and Methods. For the inhibition three concentrations of Leu20 were tested by preincubating the cells for 30 min at 4 C before addition of IL-2. As control the proliferation of TS1β cells to 1.6 U/ml of IL-9 is shown in the absence and in the presence of 15 μg/ml of IL-2 Leu20.

DISCUSSION

mAb H2-8 was isolated after immunization with IL-2 peptide 1–30. It recognizes both IL-2 and peptide 1–30, but not the shorter peptides covering the same region (Fig. 2).This result suggests that mAb H2-8 recognizes a conformational epitope on the N-terminal region of IL-2, and that this epitope is mimicked by the 1–30 peptide. Indeed, circular dichroism measurements reveal a significant fraction of α-helical structure for the 1–30 peptide. Furthermore, H2-8 can bind to peptide 1–30 even in the presence of mAb 19B11 (which recognizes a linear epitope within the non-helical part of the peptide 1–30), but does not recognize IL-2 mutants at position 20 (in the center of α-helix A) as determined by Western blot analysis or ELISA (Figs 3, 4 and data not shown). The antibody also inhibits the bioactivity of IL-2 on TS1β cells (Fig. 5), whose proliferation is strictly dependent on the expression of the human IL-2Rβ chain. Taken together, these results demonstrate that mAb H2-8 recognizes an epitope around Asp20 of IL-2, a region that directly influences the interaction of the cytokine with IL-2Rβ.

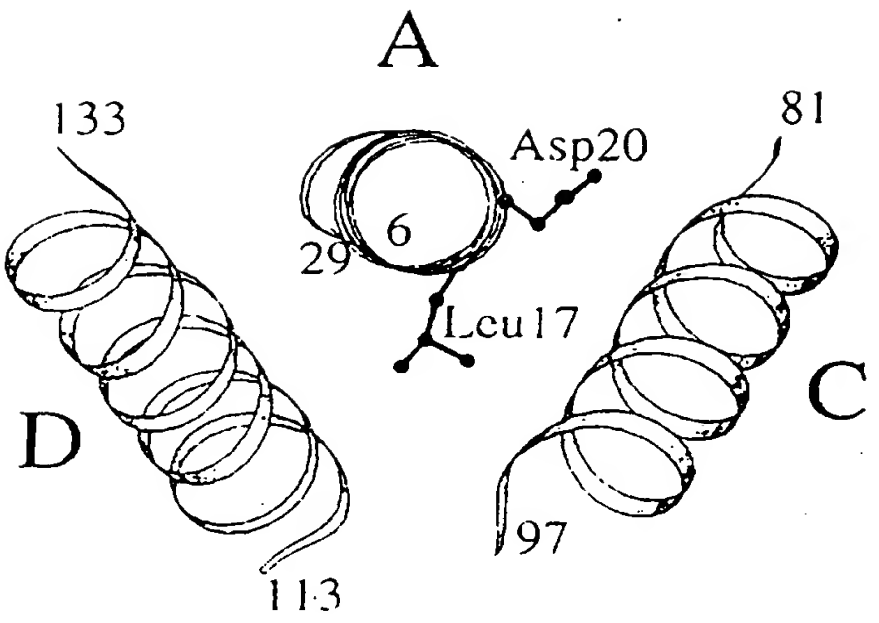
Several mutagenesis studies of IL-2 have identified amino acid substitutions that lead to deficiencies in IL-2R binding. Among these, the presence of Asp20 in helix A was initially shown to be critical for IL-2 biological activity and for effective binding to IL-2Rβ in both the human,[26,30,32] and mouse systems.[33,34] In human IL-2, mutation of Glu126 (corresponding to mouse Glu141) affects the interaction between IL-2 and IL-2Rγ.[35] According to these mutational data, the N-terminal α-helix of IL-2 (helix A) is expected to interact with the β chain of IL-2R, whereas the C-terminal α-helix D (on the opposite side of the cytokine molecule) is predicted to interact with IL-2Rγ. A possible model of these interactions based on the homologous structure of the human growth hormone and its receptor[28,36] is shown in Figure 8C. In this paper we have mainly studied the functional effect of IL-2 mutants at positions 17 and 20 of α-helix A.

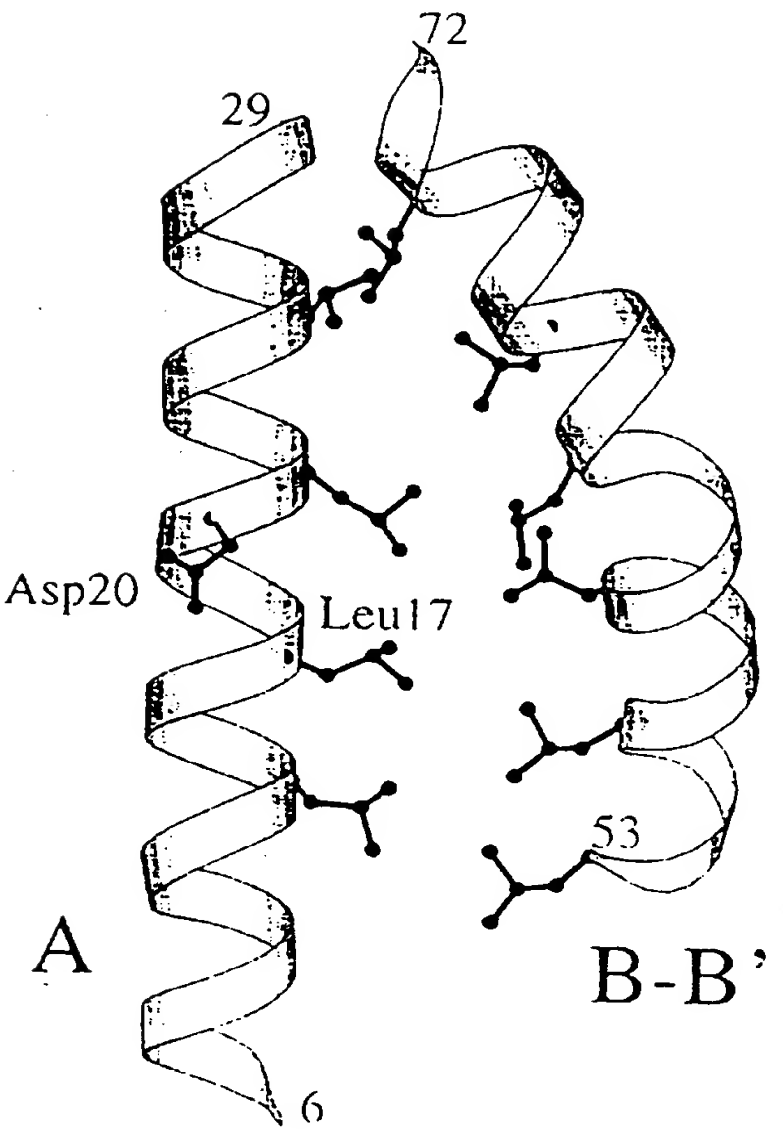
Residue Asp20 of IL-2 is located in a pocket between α-helices A and C (Fig. 8A). The carboxylate group of this amino acid is accessible to solvent and may therefore participate in direct contacts with IL-2Rβ upon formation of the cytokine–receptor complex. Indeed, the model of the cytokine–receptor complex predicts that a large fraction of the exposed surface of helix A makes contact with IL-2Rβ, with Asp20 buried within the contact interface (Fig. 8C). The carboxylate group of Asp20 may form hydrogen-bond interactions with the polar hydroxyl group of Tyr134 and/or salt-bridge interactions with the δ-guanido group of Arg15 on the β chain of the receptor. Our studies of the various mutants at position 20 are consistent with this model of interaction, since all substitutions of the carboxylate group have significant consequences on IL-2 biological activity (Fig. 6). The substitution Asp20 → Arg completely abolishes IL-2 activity. The replacement of Asp20 by Lys or Leu also has a pronounced effect on IL-2R binding, although residual activity can be measured for these mutants. In particular, the Asp20 → Leu mutant was shown to behave as partial antagonist of IL-2, being capable of inhibiting 50% of IL-2 biological activity as tested on TSβ cells (Fig. 7). Interestingly, the conservative substitution of Asp20 by the isosteric asparagine residue also produces a measurable effect on IL-2 activity, suggesting that salt-bridge interactions between IL-2 Asp20 and IL-2Rβ Arg15 may be important for efficient binding.

The results obtained with human IL-2 can be discussed in light of previous results obtained with the mouse model.[33,34] Three positions affecting IL-2–IL-2Rβ interactions were studied in the mouse. In addition to Asp34, which corresponds to Asp20 in the human molecule, two Asn residues at positions 98 and 102 in α helix C were found to be important in the biological assays. This suggests that α-helix C may be also involved in the IL-2–IL-2Rβ interactions in agreement with the location of Asp20 in the interface between α-helices A and C (Fig. 1).

Residue Leu17 is located in a leucine-rich hydrophobic environment within the protein core of IL-2. The core includes at least 10 leucine and other aliphatic side chains, most of them forming the interface between helices A and B-B' of IL-2 (Fig. 8B). Due to its internal position, the side chain of Leu17 is not likely to be involved in direct contacts with IL-2 receptor chains. However, our data demonstrate that the presence of a leucine residue at position 17 is critical for IL-2 activity. Similar results were previously obtained with mutations at position Leu21, which is also buried in the hydrophobic core of the protein.[37] These substitutions probably decrease binding by preventing proper folding or disrupting the functional conformation of IL-2.

As control we have used in this paper Cys125 → Pro and Ser127 → Pro muteins. These substitutions are expected to affect the integrity of α-helix D. However, these muteins retain a significant biological activity (Fig. 6), suggesting that the precise conformation of helix D may not be critical for interaction with IL-2Rγ. The three-dimensional model of the IL-2–IL-2Rβγ complex can explain this observation. Only a fraction of the N-terminal moiety of α-helix D is expected to be in direct contact with IL-2Rγ (Fig. 8C). Therefore, the C-terminal part of this α-helix (located at the periphery of the IL-2–IL-2Rγ interface, in a less-restrained stereochemical environment) might allow significant conformational





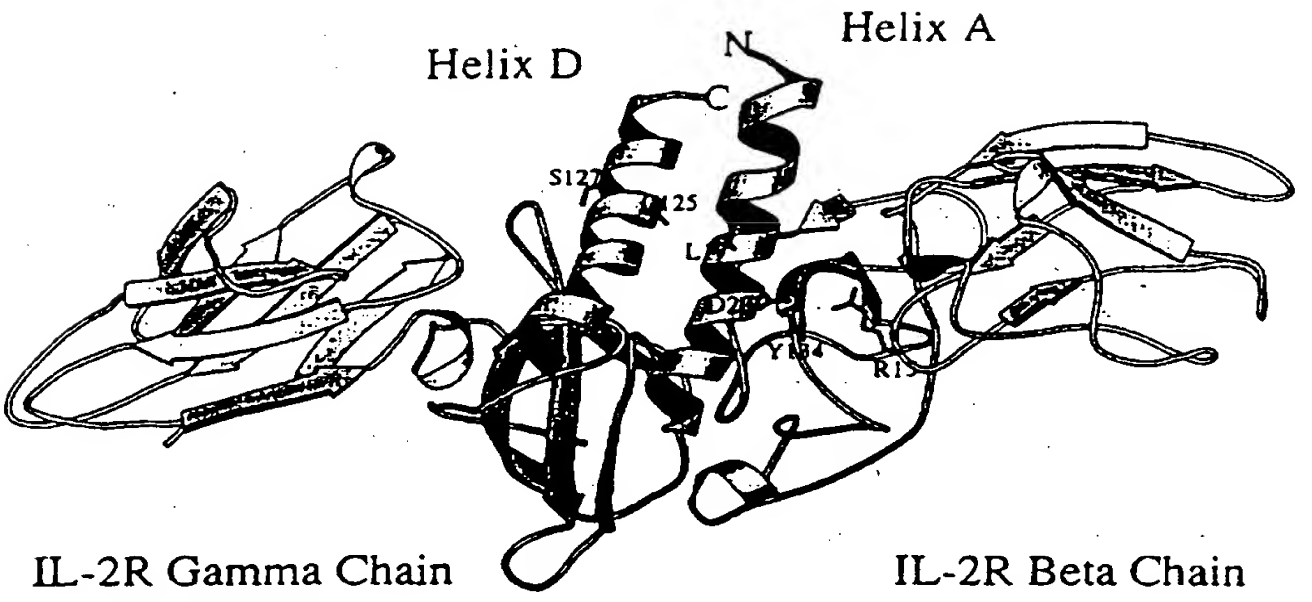


Figure 8. Model of IL-2–IL-2R interactions.

(A) Position of residues Leu17 and Asp20 in the IL-2 structure with respect to helices A, C and D, in a view perpendicular to the axis of helix A.

(B) Position of residue Leu17 with respect to helices A and B-B'. The side chain of Leu17 is located in a leucine-rich hydrophobic core of the molecule. The charged side chain of Asp20 is partly exposed to solvent. The two orientations of the molecule shown here are roughly perpendicular to that shown in Figure 1.

(C) The model of the IL-2–IL-2R complex[36] is based on the structure of the human growth hormone and its receptor.[36] For clarity, only α-helices A and D of IL-2 and the β and γ chains of the receptor are shown. The IL-2 positions studied, Leu17, Asp20 and residues Arg15 and Tyr134 of IL-2Rβ are labelled. Positions Cys125 and Ser127 are also shown. Secondary structural elements as defined by the program DSSP.[44] Atomic coordinates of the complex were obtained from the Brookhaven Protein Data Bank, entry code 1ilm.

rearrangements (such as those introduced by the insertion of proline residues within the helix) without greatly affecting receptor binding.

The data obtained with the various mutants are in agreement with the results obtained with mAb H2-8, and demonstrate the direct or indirect critical role of positions 17 and 20 of α helix A in IL-2 function. Since the effects of H2-8 and the bioactivity of all the mutants were tested on TS1β cells, the data can be interpretated as resulting from the interaction between IL-2 and IL-2Rβ. Considering the important immunoregulatory role of this cytokine and the therapeutic promise it holds for the treatment of certain cancers and infectious diseases, IL-2 has been the focus of a number of structure–function studies. The present results should help in designing new mutant proteins with agonist or antagonist activity.

MATERIAL AND METHODS

Characterization of mouse monoclonal antibody H2.8

Female Balb/c mice were repeatedly immunized with 25–50 µg of peptide 1–30 per injection. The peptide was coupled to the KLH carrier and injected with Complete Freund's adjuvant (first injection) or incomplete Freund's adjuvant (subsequent injections). The titre of the anti-IL-2 activity was assessed in a group of five animals. Spleen cells from the animal giving the best response were used for fusion with cell line SP2-0. Four hybridomas with specific anti-IL-2 activity were cloned. The mAbs were purified from the corresponding ascitic fluid by ammonium sulfate precipitation. The purity of the reagents (>80%) was verified by polyacrylamide gels. The properties of the mAbs were characterized. The results are reported only for mAb H2-8 . The isotype (IgG1) and the K_d (1.4×10^{-9} M) of mAb H2-8 were determined.

Mouse mAbs 19B11 (IgG1) and 2C4 (IgG1) previously characterized[36,37] were used as controls. mAbs 19B11 and 2C4 inhibit the binding of IL-2 to IL-2Rβ and recognize the peptides 1–10 (see below), 1–22 and 1–30. Rat monoclonal 11B11 (IgG, k) specific for murine IL-4 was provided by Dr W. Paul (National Institute of Health, Bethesda MD, and used as previously described.[39]

Human IL-2 and IL-2 muteins

Human non-glycosylated rIL-2 was provided by Roussel Uclaf (Romainville, France). For the generation of IL-2 mutants, IL-2 cDNA was cloned into M13mp18 or M13mp19 to be used as the template; site-directed mutagenesis was performed on uracil-containing single-stranded DNA template.[38] The mutated cDNA was then sequenced, and the desired mutant cDNA was cloned into the P_L promoter-based expression vector pLY-4 (Jianliang Yu *et al.*, unpublished results). Partial characterization of the mutants was performed.[39]

Peptide synthesis

Peptides were synthesized by the stepwise solid-phase reaction using the Boc/trifluoroacetic acid method,[40] on a *p*-methylbenzhydrylamine resin (Applied Biosystems) with an Applied Biosystems 430A peptide synthesizer. Tri-functional amino acids were protected as follows: Arg (tosyl), Asp (*O*-cyclohexyl ester), Glu (*O*-cyclohexyl ester), Lys (Nε-2-chlorobenzyloxycarbonyl), Ser (benzyl ether), Thr (benzyl ether), His (dinitrophenyl), Met (sulfoxide), Arg (tosyl), Tyr (2-bromobenzyl). After synthesis the Boc group was removed and the N-terminal function was acetylated with acetic anhydride. Final deprotection and cleavage of the peptidyl resin was performed by treatment with hydrogen fluoride for 1 h at 0 C in the presence of *p*-cresol. For the peptides containing a methionine residue this operation was preceded by treatment with HF/DMS to remove the sulfoxide group. The cleaved deprotected peptides were precipitated with cold diethylether, dissolved in 5% acetic acid and lyophilized. The peptides were purified by reverse-phase preparative HPLC. Following purification, peptides were verified by mass spectrometry and amino acid analysis after total hydrolysis. The following IL-2 peptides were used in the present work: 1–10, 5–15, 10–20, 15–25, 20–30, 1–22 and 1–30.

ELISA and binding inhibition

The reactivity of anti-IL-2 mAbs against rIL-2, IL-2 muteins or peptides was analysed by ELISA. Wells were coated with a solution containing 1 µg/ml of IL-2, IL-2 muteins or peptides in K_2HPO_4 KH_2PO_4, 0.05 M, pH 8 buffer. After overnight incubation at 4 C, wells were washed and saturated with BSA. Several dilutions of purified anti-IL-2 mAb were incubated for 1 h at 37 C. Wells were washed (PBS–tween 20, 0.1%) followed by the addition of an alkaline phosphatase goat anti-mouse polyvalent Ig conjugate from Sigma (St Louis, MO). After 1 h at 37 C wells were washed and substrate (Sigma 104 phosphatase substrate) was added at 1 mg/ml. The OD (405 nm) was determined after 1 h at 37 C.

For inhibition experiments a concentration of mAbs giving half maximal binding was used. This solution was mixed for 1 h at 37 C with different concentrations of inhibitor before addition to the wells. For competition experiments involving mAb 19B11 the plates were coated with mAbs H2-8, 19B11 or 3H9 (solution at 10 µg/ml, overnight incubation at 4 C). Some wells received peptide 1–30 (10 µg/ml, 1 h at 37 C) before washing. The binding of biotin-labelled mAb 19B11 was then measured by addition of alkaline phosphatase–avidin conjugate.

Western blot analysis

IL-2 mutants (50 ng) were separated by 15% SDS-PAGE and electroblotted onto nitrocellulose (0.1 µm pore size, Schleicher & Schnell, Dassel, Germany) Following overnight saturation in 5% non-fat dry milk in PBS, 1% Tween 20, nitrocellulose membranes were incubated with mouse anti-IL-2 antibodies followed by peroxidase-conjugated goat anti-mouse Ig. The blots were developed using an enhanced chemiluminescence detection system (ECL, Amersham) and the films were exposed to 1 to 2 min.

Cell lines, culture media and proliferation assay

TS1 cells express only mouse IL-2Rγ. TS1β cells were obtained after transfection of TS1 cells with human IL-2Rβ

cDNA cloned in the pdKCR expression vector kindly provided by Dr T. Taniguchi (Institute for Molecular and Cellular Biology, Tokyo University, Japan). TS1α cells were obtained after transfection of TS1 cells with human IL-2Rα cDNA cloned in pCMV4 expression vector provided by Drs W. A. Kuziel and W. C. Greene (Gladstone Institute Virol./Immunol., San Francisco CA, USA). TS1β and TS1α were previously characterized.[41] CTLL2 and YT were also used for IL-2 binding studies.

All cultures were performed in complete medium composed of RPMI 1640 (BioProducts, Walkerville, MD), 10% heat inactivated FCS (Serovial, Vogelgsun, France), 2 mM glutamine, 100 units ml penicillin, 100 μg/ml streptomycin, 50 mM 2-mercaptoethanol (2-ME). TS1β and TS1α cell lines were grown as TS1 cells in complete medium supplemented with supernatant of recombinant baculovirus expressing murine IL-9 proteins (DIB 349).[42]

TS1β cells were cultured (10^5 cells/well) in 96 wells in flat-bottomed microtitre plates with a final volume of 0.2 ml. Various concentrations of human rIL-2, IL-2 muteins or mouse rIL-9 were assayed. In order to test the inhibitory effect of mAbs, different concentrations of these reagents were mixed in the culture wells with the respective lymphokines for 30 min at low temperature before adding the cells. The inhibitory effects of mutein 20 Leu was studied by preincubating the cells (30 min at 4 C) with the indicated concentration of inhibitor before adding IL-2 or IL-9 to the wells. Cultures were pulsed with 0.5 μCi/well of [^{3}H]TdR after 36 h of incubation and harvested 15 h later.

IL-2 binding assay and inhibition

The IL-2 binding assay was performed as already described.[29] ^{125}I-labelled IL-2 binding to different cell lines was first studied. Inhibition experiments were performed at concentration of ^{125}I-labelled IL-2 giving between 50 to 70% maximum binding. The effects of the different muteins was analysed after 1-h preincubation at 4 C followed by incubation with ^{125}I-labelled IL-2 (3 h at 4 C). In each experiment non-specific binding was determined. The data were expressed as a percentage of inhibitory capacity of the different mutein vs wild-type protein.

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REFERENCES

1. Taniguchi T, Matsui H, Fujita T, Takaoka C, Kashima N, Yoshimoto R, Hamuro J (1983) Structure and expression of a cloned cDNA for human interleukin-2. Nature 302:305–310.

2. Robb RJ, Kutny RM, Panico M, Morris HR, Chowdhry V (1984) Amino acid sequence and post-translational modification of human interleukin-2. Proc Natl Acad Sci USA 81:6486–6490.

3. Smith KA (1988) Interleukin-2. Academic Press Inc., New York, USA.

4. Smith KA (1988) Interleukin-2: inception, impact and implications. Science 240:1169–1176.

5. Minami Y, Kono T, Miyazaki T, Taniguchi T (1993) The IL-2 receptor complex: its structure, function and target genes. Annu Rev Immunol 11:245–267.

6. Taniguchi T, Minami Y (1993) The IL-2/IL-2 receptor system: a current overview. Cell 73:5–8.

7. Uchiyama T, Broder S, Waldmann T (1981) A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. J Immunol 126:1293–1297.

8. Leonard WJ, Depper JM, Crabtree GR, Rudikoff S, Pumphery J, Robb RJ, Kronke M, Svetlik PB, Peffer NJ, Waldmann TA, Greene WC (1984) Molecular cloning and expression of cDNAs for the human interleukin-2 receptor. Nature 311:626–631.

9. Kumar A, Moreau J-L, Thèze J (1987) Internalization of interleukin-2 (IL-2) by high affinity IL-2 receptors is required for induction of the growth of IL-2 dependent T cell lines. J Immunol 139:3680–3684.

10. Bismuth G, Moreau J-L, Sommé G, Duphot M, Dautry-Varsat A, Robb R, Thèze J (1985) Regulation of interleukin-2 (IL-2) receptor expression: IL-2 as an inducing signal for the expression of its own receptor on a murine T helper cell line. Eur J Immunol 15:723–727.

11. Froussard P, Jankovic DL, Chastagner P, Thèze J (1991) Induction of mouse p55 interleukin-2 receptor gene expression by IL-2 and IL-4 and characterization of its transcriptional initiation sites. Mol Immunol 28:87–93.

12. Teshigawara K, Wang HM, Kato K, Smith KA (1987) Interleukin-2 high-affinity receptor expression requires two distinct binding proteins. J Exp Med 165:223–238.

13. Hatakeyama M, Tsudo M, Minamoto S, Kono T, Doi T, Miyata T, Miyasaka M, Taniguchi T (1989) Interleukin-2 receptor β chain gene: generation of three receptor forms by cloned human α and β chain cDNA's. Science 244:551–556.

14. Tsudo M, Kitamura F, Miyasaka M (1989) Characterization of the interleukin 2 receptor β chain using three distinct monoclonal antibodies. Proc Natl Acad Sci USA 86:1982–1986.

15. Takeshita T, Asão H, Ohtani K, Ishii N, Kumaki S, Tanaka N, Munakata H, Nakamura M, Sugamura K (1992) Cloning of the γ chain of the human IL-2 receptor. Science 257:379–382.

16. Ishii N, Takeshita T, Kimura Y, Tada K, Kondo M, Nakamura M, Sugamura K (1994) Expression of the IL-2 receptor γ chain on various populations in human peripheral blood. Int Immunol 6:1273–1277.

17. Thèze J (1994) Cytokine receptors: a combinative family of molecules. Eur Cytokine Netw 5:353–368.

18. Moreau J-L, Chastagner P, Tanaka T, Miyasaka M, Kondo M, Sugamura K, Thèze J (1995a) Control of IL-2 responsiveness of B lymphocytes by IL-2 and IL-4. J Immunol 155:3401–3408.

19. Chastagner P, Moreau J-L, Jacques Y, Tanaka T, Miyasaka M, Kondo M, Sugamura K, Thèze J (1996) Lack of intermediate affinity IL-2 receptor in mice leads to a dependence on IL-2 receptor α, β and γ chain expression for T cell growth. Eur J Immunol 26:201–206.

20. MacKay D (1992) Unraveling the structure of IL-2: Reply. Science 257:410–413.

21. Sauvé K, Nachman M, Spence C, Bailon P, Campbell E, Tsien W-H, Kondas JA, Hakimi J, Ju G (1991) Localization in human interleukin 2 of the binding site to the a chain (p55) of the interleukin 2 receptor. Proc Natl Acad Sci USA 88:4636–4640.

22. Wang Z, Zheng Z, Sun L, Liu X (1995) Substitutions at the Glu^{42} residue of human interleukin-2 differentially affect its binding to the α chain and the βγ complex of the interleukin-2 receptor. Eur J Immunol 25:1212–1216.

23. Voss SD, Leary TP, Sondel PM, Robb RJ (1993) Identification of a direct interaction between interleukin 2 and the p64 interleukin 2 receptor γ chain. Proc Natl Acad Sci USA 90:2428–2432.

24. Buchli P, Ciardelli T (1993) Structural and biologic properties of a human aspartic acid-126 interleukin-2 analog. Arch Biochem Biophys 307:411–415.

25. Taniguchi T (1995) Cytokine signaling through nonreceptor protein tyrosine kinases. Science 268:251-255.

26. Collins L, Tsien W-H, Seals C, Hakimi J, Weber D, Bailon P, Hoskings J, Greene WC, Toonie V, Ju G (1988) Identification of specific residues of human interleukin-2 that affect binding to the 70-kDa subunit (p70) of the interleukin-2 receptor. Proc Natl Acad Sci USA 85:7709-7713.

27. Berndt WG, Chang DZ, Smith KA, Ciardelli TL (1994) Mutagenic analysis of a receptor contact site on interleukin-2: preparation of an IL-2 analog with increased potency. Biochemistry 33:6571-6577.

28. Bamborough P, Hedgecock CJ, Richards WG (1994) The interleukin-2 and interleukin-4 receptors studies by molecular modelling. Structure 2:839–851

29. Moreau J-L, Groote DD, François C, Jacques Y, Bossus M, Tartar A, Thèze J (1995b) Characterization of a monoclonal antibody directed against the NH2 terminal area of interleukin-2 (IL-2) and inhibiting specifically the binding of IL-2 to IL-2 receptor β chain (IL-2Rβ). Mol Immunol 32:1047–1056.

30. Ju G, Collins L, Kaffka KL, Tsien W-H, Chizzonite R, Crowl R, Bhatt R, Killian PL (1987) Structure-function analysis of human interleukin-2. J Biol Chem 262:5723–5731.

31. Flemming CL, Russels SJ, Collins MKL (1993) Mutation of Asp^{20} of human interleukin-2 reveals a dual role of the p55 α chain of the interleukin-2 receptor. Eur J Immunol 23:917–921.

32. Wu Z, Johnson KW, Choi Y, Ciardelli TL (1995) Ligand binding analysis of soluble interleukin-2 receptor complexes by surface plasmon resonance. J Biol Chem 270:16045–16051.

33. Zurawski SM, Zurawski G (1992) Receptor antagonist and selective agonist derivates of mouse interleukin-2. EMBO J 11:3905–3910.

34. Zurawski SM, Jr FV, Doyle EL, Huyge B, Flaherty K, McKay DB, Zurawski G (1993) Definition and spacial location of mouse interleukin-2 residues that interact with its heterotrimeric receptor. EMBO J 12:5113–5119.

35. Landgraf BE, Goldstein B, Williams DP, Murphy JR, Sana TR, Smith KA, Ciardelli TL (1992) Recombinant interleukin-2 analogs. J Biol Chem 267:18511–18519.

36. de Vos AM, Ultsch M, Kossiakoff A (1992) Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. Science 255:306–312.

37. Rebollo A, De Groote D, Baudrihay M, Thèze J, Jankovic DL (1992) Immunochemical characterization of antigenic domains of human IL-2: spatially distinc epitopes are associated with binding to the p55 and p70 subunits of IL-2 receptor. Mol Immunol 29:119–130.

38. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A Laboratory Manual, 2nd Edition (Press C.S.H.L.), pp. 15–74.

39. Di Xu, Eckenberg R, Moreau J-L, Liu ZY, Thèze J, Bertoglio J (1995) Biological and receptor-binding activities of human interleukin-2 mutated at residues 20 Asp, 125 Cys or 127 Ser. Eur Cytokine Netw 6:237–244.

40. Merrifield RB (1963) Solid phase synthesis. The synthesis of a tetrapeptide. J Am Chem Soc 85:2149-2145.

41. Pitton C, Rebollo A, Snick JV, Thèze J, Garcia A (1993) High-affinity and intermediate-affinity forms of the human interleukin-2 receptor, expressed in an interleukin-9-dependent murine T cell line, deliver proliferative signals via differences in their transduction pathways. Cytokine 5:362–371.

42. Uyttenhove C, Simpson RJ, Van Snick J (1988) Functional and structural characterization of p40, a mouse glycoprotein with T-cell growth factor activity. Proc Natl Acad Sci USA 85:6934-6938.

43. Bernstein FC, Koetzle TF, Williams GJB, Jr EFM, Brice MD, Rodgers JR, Kennard O, Shimanouchi T, Tasumi M (1977) The protein data bank: a computer-based archival file for macromolecular structures. J Mol Biol 112:535-542.

44. Kraulis PJ (1991) MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. J Appl Crystallogr 24:946–950.

45. Kabsch W, Sanders C (1983) Dictrionary of protein secondary structure: Pattern recognition of hydrogen-bonded and geometrical features. Biopolymers 22:2577–2637.

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CHARACTERIZATION OF A MONOCLONAL ANTIBODY DIRECTED AGAINST THE NH2 TERMINAL AREA OF INTERLEUKIN-2 (IL-2) AND INHIBITING SPECIFICALLY THE BINDING OF IL-2 TO IL-2 RECEPTOR β CHAIN (IL-2Rβ)

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Abstract—An anti-human IL-2 mAb (19B11/β) was found to selectively block the binding of IL-2 to TS1β cells expressing the interleukin-2 receptor β (IL-2Rβ) without affecting binding to TS1α cells expressing the IL-2Rα receptor. It also specifically inhibits the IL-2 driven cell proliferation in TS1β cells. These observations have lead to the hypothesis that its epitope is related to an IL-2 area involved in binding with IL-2Rβ chain. This epitope was identified using various peptides covering the N-terminal half (including α helix A) of the 133 amino acids of IL-2. MAb 19B11 β does not recognize peptides 30–54 and 44–54 but recognizes peptides 1–22 and 1–30 with a good affinity. Furthermore, threonine in position no. 3 was found to be critical for the binding of mAb 19B11 β. A relationship between the epitope of mAb 19B11/β and the glycosylation of the IL-2 molecule was observed. This further demonstrates that the NH_2 terminal area of IL-2 is critical for IL-2 IL-2Rβ interactions. Two other mAbs were studied during the course of this work. They served as control for the study of mAb 19B11/β and provide some additional insight concerning the question of IL-2 IL-2R structure-function. MAb 16F11/α selectively blocks the IL-2 binding to TS1α cells. The epitope of mAb 16F11 is conformational and it was not possible to study the corresponding IL-2 IL-2Rα region of interaction. Epitope of mAb 3H9 is localized between residues 30 and 54 and does not affect the binding of IL-2 to IL-2Rα.

Key words: IL-2, IL-2 receptor, anti-IL-2 mAb.

INTRODUCTION

Interleukin-2 (IL-2) is a 133 aa polypeptide of 15–18 kDa depending on e degree of glycosylation (Bazan, 1992; Robb *et al.*, 1984; Smith, 1988; Taniguchi *et al.*, 1983). It is secreted by activated T cells and despite the increasing number of cytokines able to promote T cell proliferation (IL-4, IL-7, IL-9, IL-6, TNFα etc.), IL-2 remains the main T cell growth factor. IL-2 also modulates the activation, proliferation and differentiation of other cells in the immune system including NK-cells, B cells and monocytes (Smith, 1988).

The effects of IL-2 on these various cells are mediated through specific cell surface receptors (IL-2R) (Minami *et al.*, 1993; Taniguchi and Minami, 1993). Over the past few years, our understanding of the IL-2R complex has increased substantially. It is now known that the IL-2R comprises at least three subunits encoded by different genes. These subunits can be expressed individually or in various combinations resulting in receptors that bind IL-2 with markedly different affinity. The first IL-2R component to be identified, IL-2Rα, is a 55-kDa protein that binds IL-2 with a K_d of ~10 nM (Leonard *et al.*, 1984; Uchiyama *et al.*, 1981). The immunochemistry of IL-2Rα (Kumar *et al.*, 1987; Malek *et al.*, 1983; Moreau *et al.*, 1987) and the role of IL-2 on IL-2Rα gene expression have been studied (Froussard *et al.*, 1991; Jankovic *et al.*, 1989). The second IL-2R component, IL-2Rβ, is a 75-kDa protein with a large intracytoplasmic domain (286 aa) that plays an important role in receptor-mediated signaling (Hatakeyama *et al.*, 1989; Teshigawara *et al.*, 1987; Tsudo *et al.*, 1989). The third component recently identified, IL-2Rγ is a 64-kDa protein (Ishii *et al.*, 1994; Takeshita *et al.*, 1992). IL-2Rβ and IL-2Rγ alone have a very low affinity for IL-2. However, when expressed

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Abbreviations: aa, amino acid, IL-2, interleukin-2, IL-2R, interleukin-2 receptor, K_d, dissociation constant, mAb, monoclonal antibody.

together, these two chains form an intermediate affinity receptor with a K_d of ~1 nM. Expression of the three receptors is required to form a high affinity IL-2R (K_d ~10 pM). IL-2Rα does not exhibit homology with the cytokine receptor family while IL2Rβ and -γ belong to that family. The IL-2Rβ chain is also part of the IL-15R complex. The IL-2Rγ is common to IL-4R, IL-7R, IL-9R and IL-15R (Thèze, 1994). Heterodimerization of IL-2Rβ and IL-2Rγ is required for signal transduction. The dimer binds tyrosine kinase JAK1 and JAK3 and this represents the primary event during the IL-2-mediated T cell activation (Nakamura *et al.*, 1994).

Numerous studies employing mutagenesis have been performed to probe IL-2/IL-2 receptors interactions. In the mouse system this has led to the identification of two critical sites for IL-2 binding to IL-2Rβ and IL-2Rγ. While aspartic acid in position 34 is essential for the binding to IL-2Rβ, glutamine in position 141 is involved in binding to IL-2Rγ. Nineteen amino acids are important for IL-2 binding to IL-2Rα (Zurawski *et al.*, 1993; Zurawski and Zurawski, 1992). Much less is known about human IL-2 receptor binding. In one study a single mutation near the N-terminus of IL-2 (Asp 20) was reported to specifically perturb the interaction between IL-2 and IL-2R of intermediate affinity (Collins *et al.*, 1988; Flemming *et al.*, 1993; Ju *et al.*, 1987).

We have previously characterized a family of eight monoclonal antibodies (mAbs) able to affect differentially the binding of human IL-2 to either the α chain or the βγ complex (Rebollo *et al.*, 1992). In the present study we have analysed in more detail the properties of mAb 19B11/β able to specifically inhibit the binding of IL-2 to IL-2Rβ. We also report on the properties of mAb 16F11/α —inhibiting the binding of IL-2 to IL-2Rα — and some characteristics of mAb 3H9 that do not affect the binding of IL-2 to any of the IL-2Rs. These results extend our previous results and demonstrate that, in agreement with data from mutagenesis experiments, the NH_2 terminal part of human IL-2 is critical for binding to IL-2Rβ.

MATERIALS AND METHODS

Reagents and antibodies

Human non-glycosylated rIL-2 was a generous gift of Roussel Uclaf (Romainville, France). Human glycosylated rIL-2 produced in Chinese hamster ovary (CHO) cells was kindly provided by Dr Ferrara (Sanofi, Castanet-Tolosan, France).

Supernatant from Hela subline (H28) transfected with the plasmid pKCRIL-4 was kindly provided by Dr T. Honjo (Kyoto University, Japan) and was used as a source of murine rIL-4 (Severinson *et al.*, 1987).

The soluble β chain has been produced from RNAs extracted from YT-2C2 cells. After extraction, cDNAs has been obtained by treatment with a DNA polymerase. Two oligonucleotides, one of them specific for the domain near the initiation site, the other one specific for the sequence near the sequence coding for transmembrane domain, have been constructed and used to amplify the cDNA fragment coding for the extracellular domain of the β chain. Then, this fragment was inserted into an expression vector, pKCR6 (Matrisian *et al.*, 1986), to realise a stable transfection into CHO cells. Secreted product was purified using an immunoaffinity column with a monoclonal anti-β chain (CF1).

Mouse monoclonal antibodies 19B11/β (IgG1), 16F11/α (IgG2a) and 3H9 (IgG1) were produced as previously described (Rebollo *et al.*, 1992). Briefly, female BALB/c mice were repeatedly immunized with 5 μg of human non-glycosylated rIL-2. Splenocytes were fused with NSØ myeloma cell line. After fusion, screening for anti-IL-2 activity was carried out by immunoprecipitation in liquid phase. After cloning, the reactivity of anti-IL-2 mAbs was tested by ELISA. Isotypes of anti-11-2 mAb were determined using the kit supplied by Miles Scientific (Naperville, IL). Rat monoclonal 11B11 (IgG, k), specific for murine IL-4, was provided by Dr W. Paul (National Institute of Health, Bethesda, MD). Goat anti-mouse polyvalent immunoglobulin alkaline phosphatase conjugate was from Sigma (St. Louis, MO).

Mouse polyclonal anti-human IL-2 antibodies were prepared in the laboratory. BALB c animals were immunized by repeated injection of 50 μg of IL-2 in CFA and were regularly bled. A pool from 10 animals was used for purification.

MAbs and polyclonal antibodies were purified in two steps: ammoniac sulfate precipitation was followed by chromatofocusing as described by the supplier (Pharmacia, France).

Cell lines and culture media

TS1 cells were maintained in complete medium supplemented with supernatant of recombinant baculovirus expressing murine IL-9 proteins (DIB 349) (Uyttenhove *et al.*, 1988).

TS1β cells were obtained after transfection of TS1 cells with human p70 IL-2R CDNA cloned in the pdKCR expression vector kindly provided by Dr T. Taniguchi (Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan). TS1αβ cells were obtained after transfection of TS1β cells with human p55 IL-2R cDNA cloned in pCMV4 expression vector kindly given by Drs W.A. Kuziel and W.C. Greene (Gladstone Institute Virol./Immunol., San Francisco, CA). TS1β and TS1αβ were previously characterized (Pitton *et al.*, 1993) and TS1β and TS1αβ cell lines were grown as TS1 cells in complete medium supplemented with IL-9.

YT cells were generous gift from Dr. J. Yodoï (Kyoto University, Kyoto, Japan) and were maintained in complete medium (Yodoi *et al.*, 1985).

All cultures were performed in complete medium composed of RPMI 1640 (BioProducts, Walkerville, MD), 10% heat inactivated FCS (Serovial, Vogelgsun, France), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 50 mM 2-β-mercaptoethanol (2β-ME).

Proliferation assay

Cells were cultured (10^4 cells/well) in 96-wells plat-bottomed microtiter plates in a final volume of 0.2 ml. Different concentrations of human rIL-2 and mouse rIL-4 were tested.

Concentrations giving about half maximal proliferation were used for inhibition experiments. The same concentrations were used for TS1αβ and TS1β (rIL-2, ng/ml; IL-4, 12.5 U/ml). Serial dilution of anti-IL-2 or anti-IL4 mAb antibodies were mixed in the culture wells with the respective lymphokines 30 min before adding the cells.

Cultures were pulsed with 0.5 μCi/well of (^{3}H) TdR after 36 hr of incubation and harvested 15 hr later.

IL-2 binding assay and inhibition

The IL-2 binding assay was performed as follows. Serial dilutions of ^{125}I-labelled IL-2 were incubated with 5×10^5 cells in RPMI 1640 medium containing 10 mg/ml BSA, 25 mM Hepes and 0.2% sodium azide in a total volume of 100 μl for 30 min at 37°C. After incubation, cell-bound radioactivity was separated from free radioactivity by centrifuging the cells through a 200 μl layer of a mixture of 84% silicone oil (DC 550, Serva, Germany) and 16% paraffin oil (Neyol Laboratories, Fimouze, France) in 400 μl polyethylene tubes. The bottoms of the tubes containing the cell pellets were cut off and the radioactivity was measured.

For inhibition experiments, 2.5 ng ^{125}I-IL-2 were used per well. Serial dilution of mAbs were preincubated with ^{125}I-IL-2 during 30 min before adding the cells. The same protocol was used to study the inhibitory capacity of soluble IL-2Rβ

ELISA, binding inhibition and affinity measurements

ELISA was used to test the reactivity of anti-IL-2 mAb against rIL-2 and peptides. Wells were coated with a solution containing 1 μg/ml of IL-2 or peptides in K_2HPO_4/KH_2PO_4, 0.05 M, pH 8 buffer. After overnight incubation at 4°C, wells were washed and saturated with bovine serum albumin (BSA). Several dilutions of purified anti-IL-2 mAb (or purified polyclonal antibodies) were added for 1 hr at 37°C. Wells were washed (PBS–Tween 20, 0.1%) followed by the addition of an alkaline phosphatase anti-mouse Ig conjugate. After 1 hr at 37°C wells were washed and substrate (Sigma 104 phosphatase substrate) was added at 1 mg/ml. The O.D. (405 nm) was read in a spectrophotometer after 1 hr at 37°C.

For the inhibition experiment, a concentration of mAbs giving half maximal binding was used (19B11/β, 0.1 μg/ml; 16F11/α, 0.4 μg/ml; 3H9, 0.1 μg/ml). This solution was mixed during 1 hr at 37°C with different concentrations of inhibitor before addition to the wells.

Affinities (K_d) were calculated according to Friguet *et al.* (1985). In some experiments, IL-2 was treated by 2β-ME. IL-2 (20 μg/ml) diluted in K_2HPO_4/KH_2PO_4, 0.05 M, pH 8 buffer was mixed with different concentrations (from 0.04 M to 5 M) of 2β-ME during 1 hr at room temperature. This mixture (50 μl/wells) was directly used for coating the cells used for ELISA as described above.

Peptide synthesis

Peptides were synthesized by stepwise solid-phase method according to Boc/trifluoroacetic acid scheme (Merrifield, 1963), on a *p*-methylbenzhydrylamine resin (Applied Biosystems) in an Applied Biosystems 430A peptide synthesizer. Tri-functional amino acids were protected as follows: Arg (tosyl), Asp (*O*-cyclohexyl ester), Glu (*O*-cyclohexyl ester), Lys (Nε-2-chlorobenzyl-carbonyl), Ser (benzyl ether), Thr (benzyl ether), His (dinitrophenyl), Met (sulfoxyde), Arg (tosyl), Tyr (2-bromo-benzyl). At the end of the synthesis the Boc group was removed and the N-terminal function was acetylated with acetic anhydride. Final deprotection and cleavage of the peptidyl resin was performed by treatment with hydrogen fluoride for 1 hr at 0°C in the presence of *p*-cresol. For the peptides containing a methionine, this operation was preceded by treatment with HF DMS to remove the sulfoxyde group. The cleaved deprotected peptides were precipitated with cold diethylether, dissolved in 5% acetic acid and lyophilized.

Crude peptides were purified by reverse-phase preparative HPLC. After purification, peptides were checked for identity by mass spectrometry and amino acid analysis after total hydrolysis.

Pepscan

The overlapping 10-mer peptides spanning the 133 amino acids of the IL-2 sequence were prepared using Pepscan on polyethylene rods according to the procedure described by Geysen *et al.* (1984). At the end of the synthesis, the amino-terminal protecting group was removed and the peptides were acetylated using acetic anhydride.

Binding of the mAb to the different decamers was performed as described in the ELISA section.

RESULTS

Inhibition of binding of radiolabelled IL-2 by mAbs

To assay the effects of mAb 19B11/β on IL-2 binding, two cell types were used. These two cell clones (TS1α and TS1β) were isolated from the same mouse T cell (TS1) after transfection either by human IL-2Rα gene or IL-2Rβ gene. Since TS1 cells spontaneously express only mouse IL-2Rγ (γ_{mu}) they do not bind radiolabelled IL-2. Therefore, the IL-2 binding observed with TS1α and TS1β is strictly dependent on the presence of human IL-2R α_{hu} or IL-2Rβ_{hu}.

MAb 19B11/β specifically inhibits the IL-2 binding to TS1β while mAb 16F11/α or 3H9 have no effect on this binding (Fig. 1). However, even at high concentrations of 19B11/β, the inhibition was not complete. On TS1α cells only mAb 16F11/α inhibit completely the binding. Surprisingly, with TS1α cells mAb 19B11/β and 3H9 enhance the IL-2 binding in a narrow range of con-

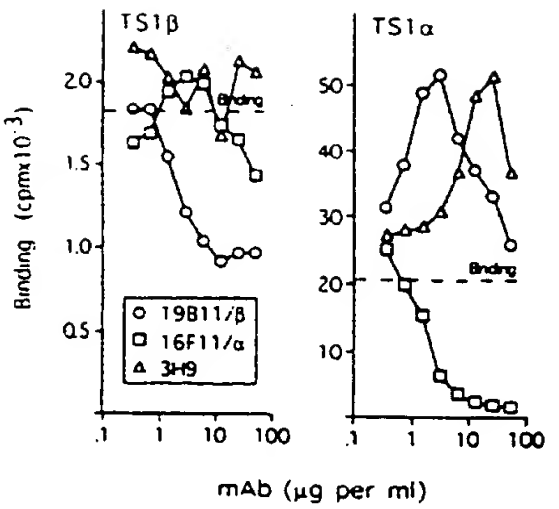

Fig. 1. Inhibition of IL-2 binding by mAb. The IL-2 binding assay was performed as described in Materials and Methods using TS1α cells (IL-2Rα$_{hu}$γ$_{mu}$) or TS1β cells (IL-2Rβ$_{hu}$γ$_{mu}$). The effect of serial dilutions of mAbs 19B11/β (-○-○-), 16F11/α (-□-□) and 3H9 (-△-△-) was assayed on the binding of 2.5 ng of ^{125}I-IL-2 to 5 × 10^5 cells. The binding obtained without inhibitor (——Binding) was 1800 cpm and 21 000 cpm for TS1β and TS1α cells, respectively. The results obtained in a representative experiment are shown.

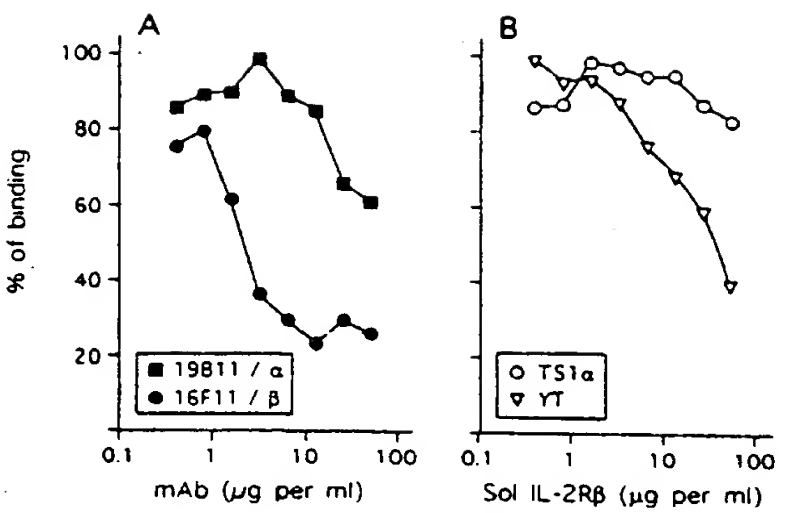

Fig. 2. Inhibition of IL-2 binding by mAb and soluble IL-2Rβ. The IL-2 binding assay was performed as described in Materials and Methods and using TS1α cells (IL-2Rα$_{hu}$γ$_{mu}$) or YT cells (IL-2Rβ$_{hu}$γ$_{mu}$). (A) The effect of serial dilution of mAbs 19B11/β (-●-●-) and 16F11/α (-■-■-) was assayed on the binding of 2.5 ng of ^{125}I-IL-2 to 5 × 10^5 YT cells. (B) The effect of different concentrations of soluble IL-2Rβ was assayed on the binding of 2.5 ng of ^{125}I-IL-2 to 5 × 10^5 TS1α cells (-○-○-) or YT (-▽-▽-). The results obtained with the two cell lines and the two inhibitors are expressed as % of binding without inhibitor.

centrations (Fig. 1). This effect could be attributed to a conformational change induced on IL-2 by mAb 16F11/α and 3H9 that would enhance binding to the hybrid α$_{hu}$γ$_{mu}$ IL-2α. Alternative explanations are discussed below.

One can conclude that mAb 19B11/β specifically inhibits the binding of human IL-2 to human IL-2Rβ while mAb 16F11/α inhibits the binding to human IL-2Rα. This is in keeping with our previous observations (Rebollo *et al.*, 1992).

Inhibition of binding of radiolabelled IL-2 by soluble IL-2Rβ

The inhibitory capacity of mAb 19B11/β was verified on human YT cells which express spontaneously the human β and γ IL-2R but not the α receptors and thus resemble to TS1β but the γ receptor is from human origin. In these cells, the inhibitory capacity of mAb 19B11/β reaches 80% under the experimental conditions used. Under the same experimental conditions, mAb 16F11/α does not inhibit significantly the binding (Fig. 2).

Using YT cells we have compared the effects of mAb 19B11/β with the effects of soluble IL-2Rβ. As expected, soluble IL-2Rβ does not inhibit the binding of radiolabelled IL-2 to TS1α cells but does partially inhibit the binding of radiolabelled IL-2 to YT cells (Fig. 2).

MAb 19B11/β has inhibitory properties comparable to IL-2Rβ and therefore may bind to a similar region of the IL-2 molecule.

Neutralization of IL-2 proliferation by mAb 19B11/β

TS1β cell line was shown to proliferate in a dose-dependent manner in response to IL-2. This response is strictly independent on the expression of IL2Rα receptor (Pitton *et al.*, 1993). This cell also proliferates in response to IL-4 or IL-9. TS1αβ cell line was obtained after transfection of TS1β with human IL2Rα gene. This cell line is dependent on the expression of IL-2Rα for IL-2 dependent cell proliferation (Pitton *et al.*, 1993). Using these cell lines the effects of mAb 19B11/β and 16F11/α and 3H9 were assayed on the IL-2 dependent proliferation.

The proliferation of TS1β was very sensitive to the effect of mAb 19B11/β (Fig. 3). This inhibition is probably related to the inhibition of IL-2 binding. However,

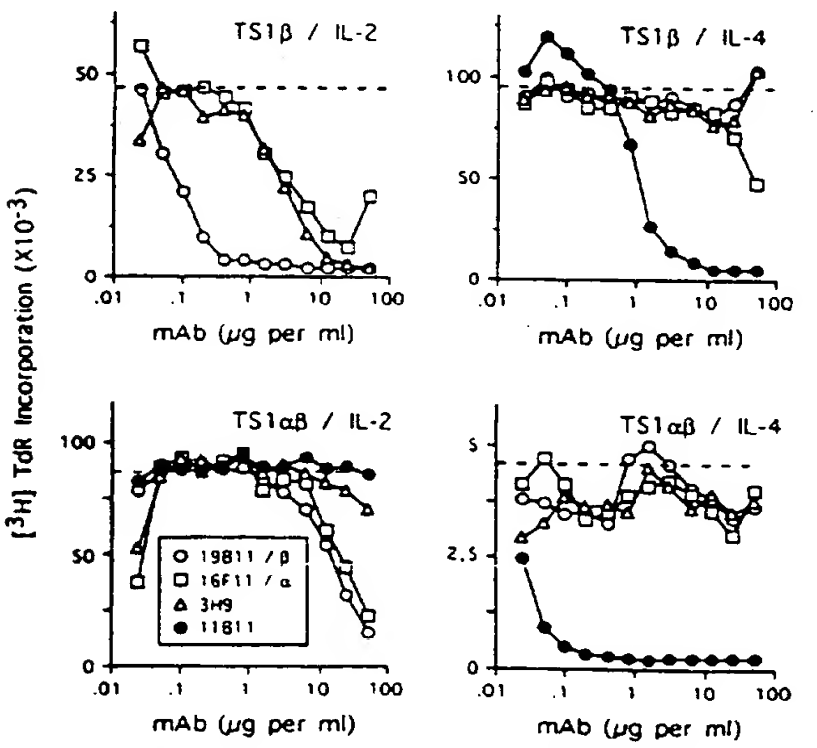

Fig. 3. Inhibition of IL-2 proliferation of TS1β and TS1αβ. The proliferation assay was conducted as described in Materials and Methods using 10^4 cells/well in a final volume of 0.2 ml. For the inhibition experiment 1 ng/ml of human rIL-2 or 12.5 U/ml mouse rIL-4 were used. The proliferation of TS1β or TS1αβ obtained with these doses of lymphokines is indicated by the dashed line. For each cell line the effects of different concentrations of mAbs is shown: -○-○-19B11/β; -□-□-16F11/α; -△-△-3H9 and - - -11B11. The result of a representative experiment is shown.

at 100-fold higher concentrations, mAb 16F11/α and 3H9 also affect the proliferation of TS1β. This effect may be related to an inhibition of internalization of the complexes formed by IL-2 and IgG molecules. The specificity of these effects is shown by the fact that the three mAbs have no significant effects on the IL-4 proliferation of TS1β under conditions in which mAb 11B11 (anti-mouse IL-4) gives a complete inhibition (Fig. 3).

The proliferation of TS1αβ is inhibited by mAb 19B11/β as well as by mAb 16F11/α thus confirming that this line is dependent on the expression of high affinity IL-2R for proliferation and that mAb 19B11 which affect the binding to IL-2Rβ also affects the biological response. As with TS1β cell line, the IL-4 proliferation of TS1αβ is not affected by the three anti-IL-2 mAbs (Fig. 3).

One may notice that 0.05 μg/ml of mAb 19B11/β is necessary for 50% inhibition of the proliferation of TS1β while 10 μg/ml is necessary for the same inhibition of TS1αβ. These 200-fold differences are explained by the difference in affinity between the β; IL-2R (10^{-9} M) and the αβ;IL − 2R (10^{-11}–10^{-12} M).

Effects of disulfide bond reduction on IL-2 recognition by mAbs

The presence of the disulfide bound between cysteine 58 and cysteine 105 has been shown to be critical for IL-2 structure and biological activity. The effect of 2-β-mercaptoethanol (2β-ME) on the conformation of the epitopes recognized by mAb 19B11/β, 16β/α and 3H9 was assayed. Mouse polyclonal antibodies made against human IL-2 were also used in these experiments.

Under the experimental conditions described in Materials and Methods, treatment with 2β-ME affects neither the binding of mAb 19B11/β nor the recognition of IL-2 by polyclonal antibodies or mAb antibody 3H9. Under the same conditions, the binding of mAb 16F11/α is greatly affected by 2β-ME treatment (Fig. 4).

This preliminary experiment suggests that the epitope recognition by mAb 16F11/α is highly conformational and is not likely to be mimicked by linear peptides. In contrast, the results obtained with mAb 1911/β suggest that it is possible to pursue the characterization of a linear peptide involved in its epitope.

Localization of the epitope of mAb 19B11/β

Four peptides were synthetized to assay the specificity of mAb 19B11/β. Peptides 1–22 and 1–30 cover the NH_2 terminal tail of IL-2 and either partially (1–22) or totally (1–30) the first α helix (α helix A). Peptides 30–54 covers all the loop joining α helix A to α helix B-B'. Peptide 44–54 covers an area including peptide 44–46 which may fold in a β-sheet fashion.

Figure 5 shows that mAb 19B11/β recognize very specifically peptides 1–22 and 1–30. No signal was obtained in ELISA on peptides 30–54 and 44–54. As expected, mAb 16F11.α does not bind to any of these peptides even though it binds to IL-2.

In the same assay polyclonal antibodies bind strongly to peptides 1–30 thus suggesting that this part of IL-2 if

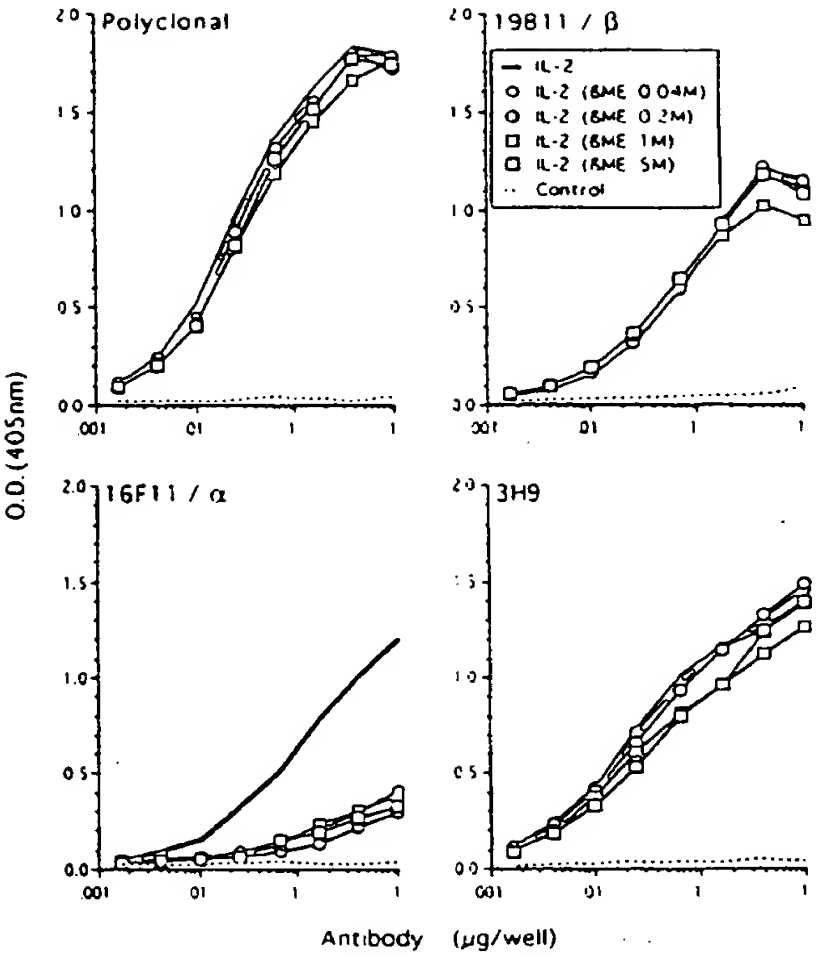

Fig. 4. Effect of β-mercaptoethanol treatment of IL-2 on the binding of polyclonal and mAbs. Before coating the plates a solution of IL-2 (20 μg/ml) was treated by different concentrations of β-ME (-○-○-: 0.04 M, -○-○: 0.2 M, -□-□-: 1 M, -□-□-5 M) during 1 hr at room temperature. Fifty microliters of these solutions were used to coat the plates. The ELISA technique used to detect the binding of purified polyclonal and mAb antibodies to β-ME-treated IL-2 is described in Materials and Methods. The results are directly given as the O.D. obtained after 1 hr incubation at 37 C.

very immunogenic. They bind less efficiently to 1–22 and 30–54 while the binding to 44–54 is very weak. Contrary to mAb 19B11/β, mAb 3H9 binds to peptide 30–54 thus showing the specificity of the system. Surprisingly, mAb 3H9 binds peptide 1–22 but more weakly peptide 1–30 although it completely encompasses the 1–22 sequence (Fig. 5). Inhibition experiments have however confirmed the full specificity of the system (see below).

Peptide inhibition of IL-2 binding and affinity measurements

Different concentrations of peptides were used to inhibit the IL-2 binding by mAbs 19B11/β, 16F11/α and 3H9 using the technique described in Materials and Methods (Fig. 6).

The binding of 19B11/β was inhibited only by peptides 1–22 and 1–30, thus confirming the binding results. The binding of 3H9 was efficiently inhibited only by peptides 30–54 and 44–54. These results confirm the complete specificity of the system. As expected, IL-2 binding by 16F11/α was not affected by these peptides.

From these data the affinities for the different peptides were calculated (see Materials and Methods and Table 1). MAb 19B11/β has a good affinity for IL-2 (K_d 2 ×

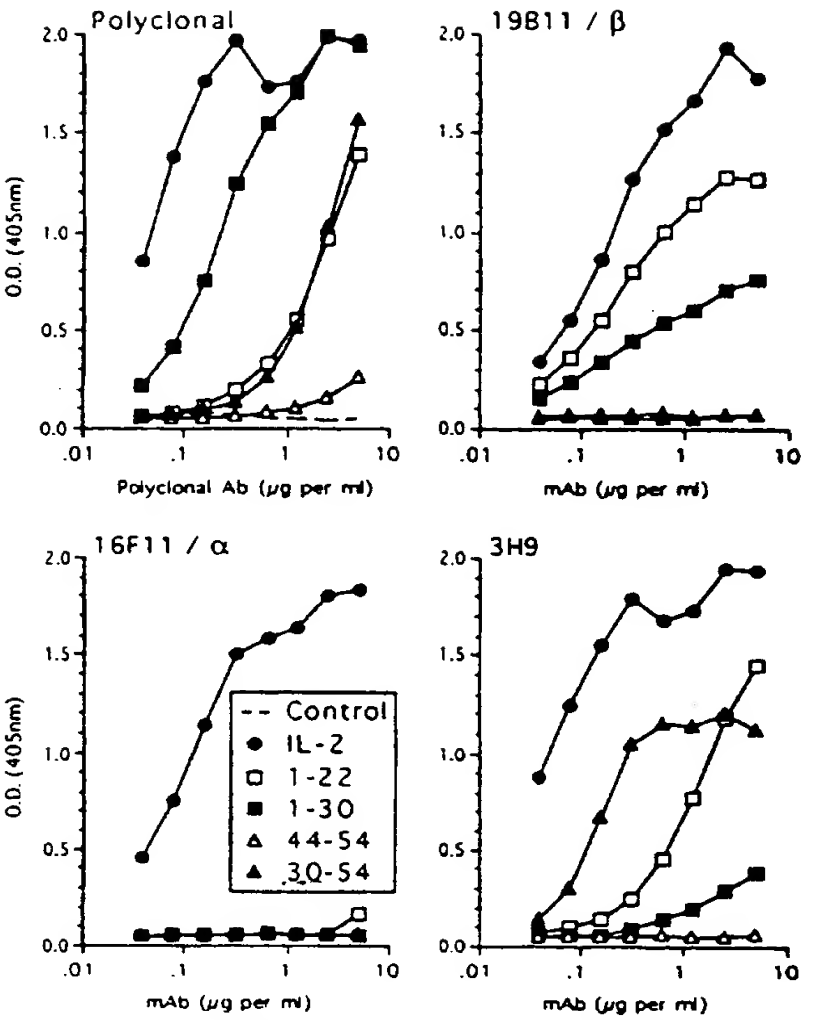

Fig. 5. Binding of mAbs to IL-2 peptides. The ELISA plates were first coated with IL-2 (-●-●-), peptide 1–22 (-□-□-), peptide 1–30 (-■-■-), peptide 44–54 (-△-△-) or peptide 30–54 (-▽-▽-) using a solution at 1 μg/ml. The ELISA technique used thereafter is described in Materials and Methods. The results obtained with the purified polyclonal antibodies and purified mAbs 19B11/β, 16F11/α and 3H9 are expressed as the O.D. obtained after 1 hr incubation at 37°C.

10^{-9} M) and peptide 1–30 (K_d 5 × 10^{-7} M). Surprisingly, mAb 3H9 has a stronger affinity for peptide 30–54 (K_d 1.3 × 10^{-9} M) than for IL-2 (K_d 1.5 × 10^{-8} M). This situation where the antibodies have a stronger affinity for a molecule not used as an immunogen has already been observed (heteroclicity).

Importance of threonine in position 3 in the epitope of mAb 19B/11β

The peptide which shows the strongest affinity for mAb 19B11/β corresponds in the folded IL-2 to a tail of 10 aa and an α helix of 20 aa. Using the pepscan method described in Materials and Methods and a series of decapeptides, we have evaluated the role of each aa in binding of 19B11/β to peptide 1–30. Figure 7 shows these results. As controls, mAbs 16F11/α and 3H9 do not bind to the series of decapeptide examined here. In contrast, mAb 19B11 binds very strongly to peptides 1–10. It also binds to peptides 2–11 and 3–12 but the loss of threonine 3 in peptide 4–13 leads to the disappearance of decapeptide recognition and no binding is observed.

Since threonine 3 is known to be the unique site of IL-2 glycosylation, we have compared the recognition of glycosylated IL-2 and non-glycosylated IL-2 by mAb 19B11/β. In ELISA, mAbs 16F11/α and 3H9 recognized these two molecules in a comparable manner. In contrast, the pattern of recognition by mAb 19B11/β is dramaticly different; this mAb does not recognize glycosylated IL-2. This is in agreement with the fact that non-glycosylated IL-2 was used for the production of mAb 19B11/β (Fig. 8).

DISCUSSION

Considering the important immunoregulatory role of IL-2, and the therapeutic promise it holds for the treatment of certain cancers and infection diseases, this cytokine has been the focus of several structure–function studies aimed at characterizing agonists, antagonists of the molecules.

A major step towards this goal has been given by the determination of the three-dimensional structure of IL-2 (Bazan, 1992). IL-2 as several other cytokines, including human growth hormone and prolactin, and these have structures composed of a compact core bundle of four antiparallel α helices. For human IL-2, helix A extends from aa 11 to 27, helix B-B′ from aa 55 to 74, helix C from aa 84 to 97 and helix D from aa 115 to 133. These helices are connected by three loops: A-B (aa 28–54); B-C (aa 75–83) and C-D (aa 98–114). Therefore, Asp 20 which is critical for binding to the IL-2R of intermediate affinity is localized in the middle of α helix A. In a more recent and detailed study the role of the segment 17–21 (Leu-Leu-Leu-Asp-Leu) surrounding Asp 20 has been investigated. It was found that Asp 20 and Leu 21 are the functionally most important residues in this region. Asp 20 is solvent accessible and likely plays a direct receptor contact role. Leu 21, in contrast, is completely buried in the hydrophobic area of the protein and substitutions at this position perturbs the hydrophobic packing arrangements important for biological activity (Berndt *et al.*, 1994).

Several lines of evidence demonstrate that mAb 19B11/β specifically inhibits the binding of human rIL-2 to IL-2Rβ.

(1) Using the same mouse T cell (TS1) —expressing only mouse γ chain — transfected either with human IL-2Rα or human IL-2Rβ, it has been possible to obtain cell lines which bind IL-2 through either the receptor IL-$2R\gamma_{mu}$/IL-$2R\alpha_{hu}$ (TS1α) or the receptor IL-$2R\gamma_{mu}$/IL-$2R\beta_{hu}$ (TS1β). Under the experimental conditions discussed in this paper, only mAb 19B11/β inhibit the binding to TS1β.

(2) This has been further documented by showing that mAb 19B11/β and soluble IL-2Rβ inhibit the IL-2 binding to human YT cells expressing the human IL-2Rβγ. Neither of these two molecules, however, give a complete inhibition of the binding and the respective amounts required for half maximal inhibition is compatible with the fact that soluble IL-2Rβ exhibits a low affinity for IL-2 (K_d 10^{-7} M).

(3) Functionally 19B11/β inhibits the growth effects of IL-2-mediated either by the intermediate affinity receptor complex (βγ) or by the high affinity receptor complex

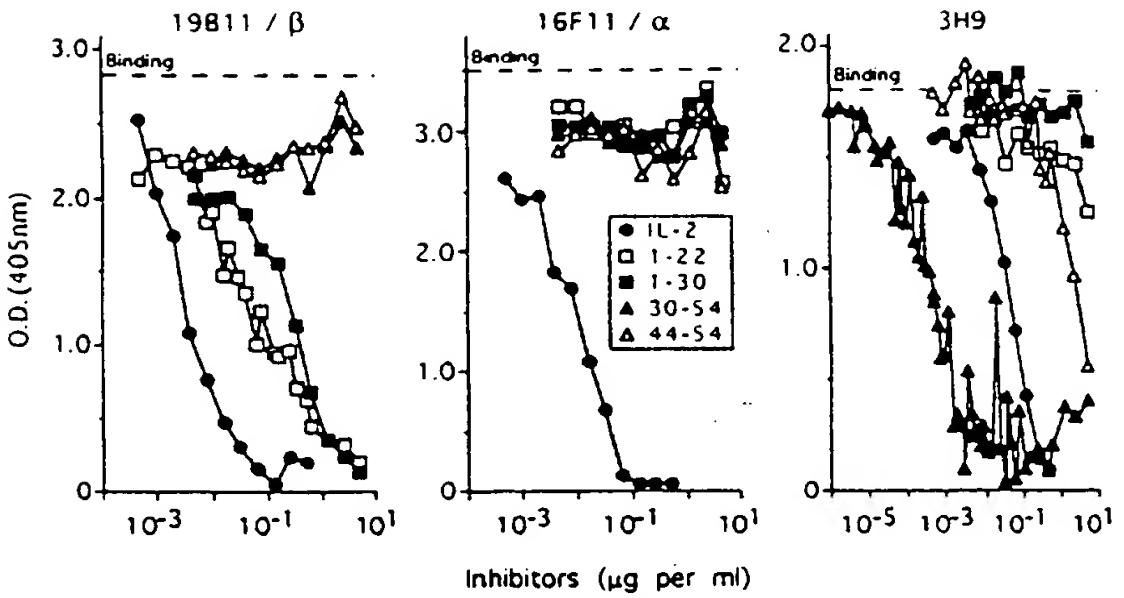

Fig. 6. Inhibition of IL-2 binding to mAbs by IL-2 peptides. These experiments were performed using the ELISA technique described in Materials and Methods. The plates were coated with rIL-2 (1 μg/ml). Concentration of mAb antibodies giving half maximal binding were used (19B11/β and 3H9 at 0.1 μg/ml; 16F11 α at 0.4 μg/ml). The corresponding binding is shown in each panel of the figure (see dashed line). The antibodies were mixed with various concentrations of these different peptides before addition to the plates: IL-2 (-●-●-), peptide 1–22 (-□-□-), peptide 1–30 (-■-■-), peptide 30–54 (-▲-▲-) and peptide 44–54 (-△-△-). The affinities of IL-2 and of the different peptides were calculated from this data (see Results and Table 1).

Table 1. Affinity of the mAb for IL-2 or IL-2 peptides (Kd in M)[a]

	IL-2	1–22	1–30	30–54	44–54
19B11/β	2.1×10^{-9}	1.3×10^{-7}	4.9×10^{-7}	$>10^{-5}$	$>10^{-5}$
16F11/α	3.2×10^{-9}	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$
3H9	1.4×10^{-9}	4.5×10^{-6}	$>10^{-5}$	1.3×10^{-9}	5.3×10^{-6}

[a] Affinities were calculated from the data represented in Fig. 7 using the method described by Friguel *et al.* (35).

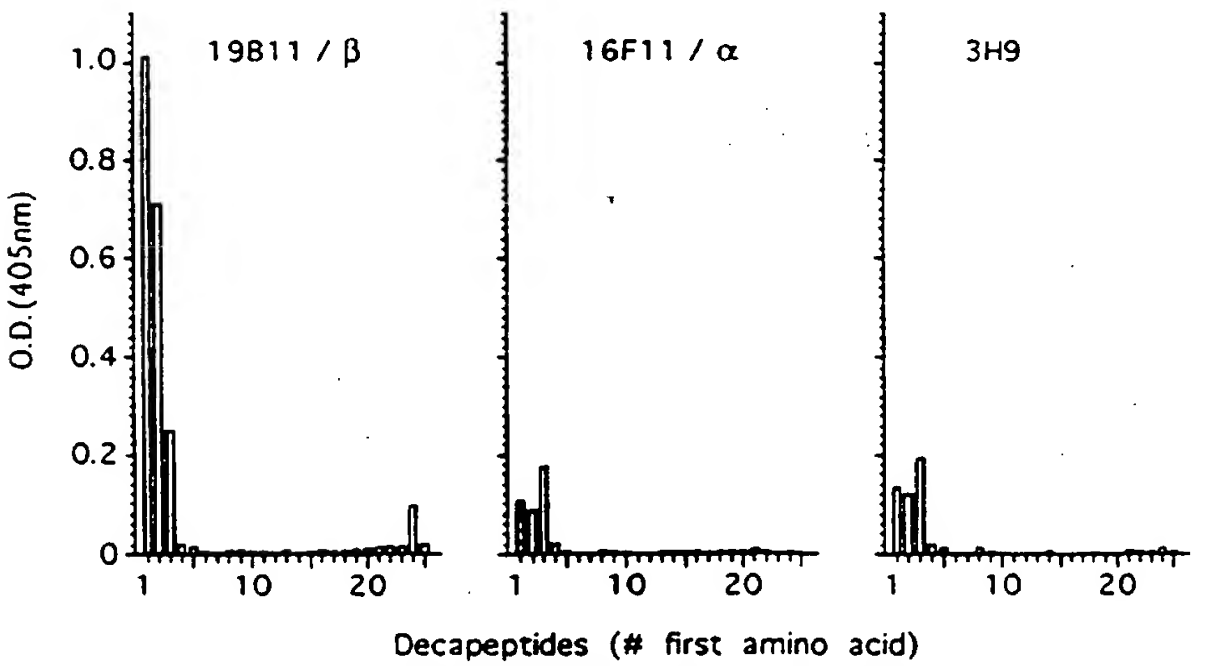

Fig. 7. Pepscan analysis of mAbs binding. The overlapping 10-mer peptides spanning the 133 amino acids of the IL-2 sequence were prepared using Pepscan on polyethylene rod as described in Materials and Methods. The binding of the mAb antibodies was then assayed using the ELISA technique described in Materials and Methods. The data are represented only for the first 25 decamers.

($\alpha\beta\gamma$). The pattern of inhibition and the dose of 19B11/β required is compatible with a blockade of the interaction between IL-2 and the IL-2Rβ chain.

(4) All these effects have been shown to be specific since mAb 16F11/α, which specifically affects the binding of radiolabelled IL-2 to rIL-2α, has been used as control.

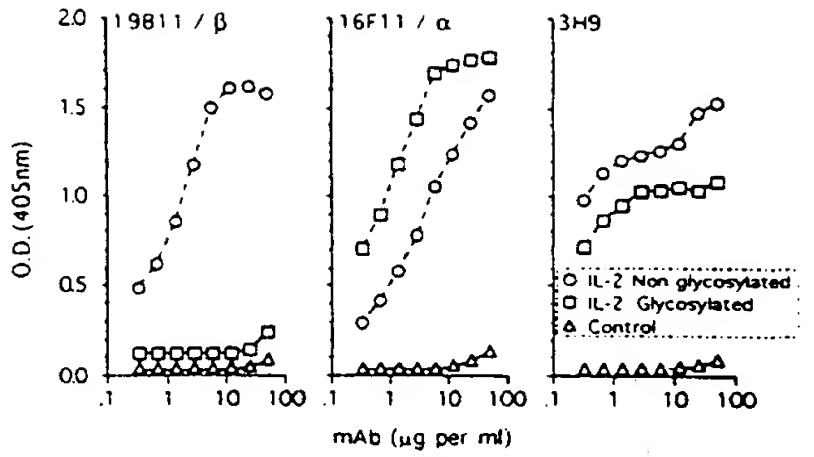

Fig. 8. Binding of mAbs to glycosylated or non-glycosylated rIL-2. The plates were coated either with glycosylated rIL-2 produced in CHO cells (Sanofi) or non-glycosylated IL-2 produced in *E. coli* (Roussel Uclaf). The binding was then analysed with the ELISA technique discussed in Materials and Methods.

MAb 16F11/α only blocks the binding to TS1α cells and not to TS1β, it does affect the binding of IL-2 to YT cells and only inhibits the IL-2 induced proliferation of TS1αβ cells. In addition, mAb 3H9 also used as control, further demonstrates the specificity of the effects observed with mAb 19B11/β.

The epitope recognized by mAb 19B11/β has been characterized using different peptides. It recognizes two peptides, namely 1–22 and 1–30 covering the NH_2 terminal part of IL-2 including the first 10 aa of the NH_2 terminal and either all (1–30) or about half of the α helix A (1–22). Its binding is totally specific and mAb 19B11/β does not recognize peptides 30–54 and 44–54. These data have been obtained by direct binding in ELISA and by inhibition experiments. The affinity of the two peptides (1–22 and 1–30) has been measured (K_d is between 10^{-7} M and 5×10^{-7} M). These data are in complete agreement with the observation that aa at positions 20 and 21 (Asp 20 and Leu 21) play an important role in IL-2/IL-2Rβ interaction.

The fine localization of the epitope recognized by mAb 19B11/β gives further insight into the interaction between IL-2 and IL-2β. Asp 20 and Leu 17 are not directly implicated in the 19B11/β epitope since various mutants of IL-2 with single aa substitution as well as double mutant at these positions are recognized as well as wild-type IL-2 by mAb 19B11/β (data not shown). The strong implication of threonine 3 was unexpected. This area of the molecule is not thought to have a well defined structure and is likely not to be the first site of interaction between IL-2 and IL-2β. The inhibitory effect of mAb 19B11/β on the interaction between IL-2 and IL-2Rβ may be due to either steric hindrance or to interaction with a secondary site of binding necessary for the stabilization of the IL-2/IL-2Rβ complex.

The observation that mAb 19B11/β does not recognize glycosylated IL-2 is in contradiction with the fact that it can, however, neutralize the biological activity of IL-2 produced *in vitro* by human peripheral lymphocytes stimulated by anti-CD3 mAb or phytohemaglutinin (data not shown). To explain this contradiction, one has to speculate that the epitope recognized by 19B11/β is localized in peptide 1–30, that threonine 3 plays a critical role in ELISA recognition but that in liquid phase other aa not hidden by the sugar moeity are accessible to the mAb and also play an important role in epitope structure.

The characterization of mAb 16F11 α and 3H9 have also provided some information. These mAbs recognize different epitopes and do not compete for the same site in IL-2 (Rebollo *et al.*, 1992). Unfortunately the epitope of mAb 16F11/α is conformational (Fig. 4) and therefore it was not possible to study this epitope related to the IL-2/IL-2Rα area of interaction. The epitope of mAb 3H9 was characterized. Its affinity for IL-2 and peptides 30–54 (A-B loop) and 44–54 is interesting to consider. Since mAb 3H9 has some affinity for peptide 44–54, one may speculate that part of its epitope is in this area even if additional aa from peptide 30–44 increases its affinity. This result can be compared to the data reported by Sauvé *et al.* (1991) showing the importance of Lys 35, Arg 38, Phe 42 and Lys 43 in IL-2/IL-2Rα interactions. MAb 3H9 does not inhibit significantly neither IL-2 binding to TS1α nor the IL-2 proliferation mediated by high-affinity IL-2 receptor (Fig. 3) maybe because of its relatively low affinity (Table 1). Furthermore, the observation showing that mAb 3H9 has greater affinity for peptide 30–54 than for IL-2 may indicate that the animals are immunized by a fragment of IL-2 produced after *in vivo* degradation or/and that in the IL-2 molecule this peptide has a conformation not easily accessible to the mAb 3H9.

Several observations made during the course of this work deserve some additional comments.

The high reactivity of polyclonal antibody from IL-2 immunized animals with peptide 1–30 shows that this area of the molecule is very immunogenic. This is independent of the glycosylation of the molecule since it has been shown that IL-2 produced in *E. coli* or in CHO cells are equally immunogenic (Sanofi, pers. comm.).

In Fig. 1 it has been shown that mAb 19B11/β stimulates the binding of radiolabelled IL-2 to TS1α cells. The work carried out in the mouse system shows that the loop including aa 30–54 as well as the α helix B-B′ plays an important role in the IL-2 binding to the α chain. One may speculate that the complex made by mAb 19B11/β and IL-2 is more stable and binds with more affinity to IL-2Rα. Alternatively, the avidity of IL-2 for IL-2Rα may be increased by the aggregation of two molecules of IL-2 on the surface of the IgG molecule. These effects also found with mAb 3H9 are dose-dependent and difficult to study.

The fact that mAb 3H9 binds peptides 1–22 and 1–30 in ELISA while it has no affinity for this peptide and the observation that on the contrary, it has significant affinity for peptide 44–54 while it does not recognize it in ELISA, further points to the necessity of measuring affinities before making any kind of conclusion on antibody–antigen interactions.

Mutagenic analysis of human IL-2 has already provided some information concerning the role of α helix A on the binding of this cytokine to the IL-2Rβ (receptor

of intermediate affinity in conjunction with the γ chain). The characterization of the epitope defined by mAb 19B11-β with specific inhibitory and functional properties described in the present paper provides complementary information. A detailed understanding of the biochemical mechanisms and structure–activity relationships of IL-2 and its receptor is required before it is possible to understand in more detail the fundamental properties of this cytokine and to develop new analogs for therapeutic applications.

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REFERENCES

Bazan J. F. (1992) Unraveling the structure of IL-2. *Science* 257, 410–413.

Berndt W. G., Chang D. Z., Smith K. A. and Ciardelli T. L. (1994) Mutagenic analysis of a receptor contact site on interleukin-2: preparation of an IL-2 analog with increased potency. *Biochemistry* 33, 6571–6577.

Collins L., Tsien W.-H., Seals C., Hakimi J., Weber D., Bailon P., Hoskings J., Greene W. C., Toome V. and Ju G. (1988) Identification of specific residues of human interleukin 2 that affect binding to the 70-kDa subunit (p70) of the interleukin 2 receptor. *Proc. natn. Acad. Sci. U.S.A.* 85, 7709–7713.

Flemming C. L., Russell S. J. and Collins M. K. L. (1993) Mutation of Asp20 of human interleukin-2 reveals a dual role of the p55 α chain of the interleukin-2 receptor. *Eur. J. Immun.* 23, 917–921.

Friguet B., Chafotte A. F., Djavadi-Ohaniance L. and Goldberg M. E. (1985) Measurements of the true affinity constant in solution of antigen–antibody complexes by enzyme-linked immunosorbent assay. *J. immunol. Meth.* 77, 305–319.

Froussard P., Jankovic D. L., Chastagner P. and Thèze J. (1991) Induction of mouse p55 interleukin-2 receptor gene expression by IL-2 and IL-4 and characterization of its transcriptional initiation sites. *Molec. Immun.* 28, 87–93.

Geysen H., Meloen R. H. and Barteling S. J. (1984) Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. *Proc. natn. Acad. Sci. U.S.A.* 81, 3998–4002.

Hatakeyama M., Tsudo M., Minamoto S., Kono T., Doi T., Miyata T., Miyasaka M. and Taniguchi T. (1989) Interleukin-2 receptor β chain gene: generation of three receptor forms by cloned human α and β chain cDNAs. *Science* 244, 551–556.

Ishii N., Takeshita T., Kimura Y., Tada K., Kondo M., Nakamura M. and Sugamura K. (1994) Expression of the IL-2 receptor γ chain on various populations in human peripheral blood. *Int. Immun.* 6, 1273–1277.

Jankovic D. L., Gibert M., Baran D., Ohara J., Paul W. E. and Theze J. (1989) Activation by IL-2, but not by IL-4, upregulates the expression of the p55 subunit of IL-2 receptor on IL-2 and IL-4 dependent T cell lines. *J. Immun.* 142, 3113–3120.

Ju G., Collins L., Kaffka K. L., Tsien W.-H., Chizzonite R., Crowl R., Bhatt R. and Killian P. L. (1987) Structure–function analysis of human interleukin-2. *J. biol. Chem.* 262, 5723–5731.

Kumar A., Moreau J. L. and Thèze J. (1987) Internalization of interleukin-2 (IL-2) by high affinity IL-2 receptors is required for induction of the growth of IL-2 dependent T cell lines. *J. Immun.* 139, 3680–3684.

Leonard W. J., Depper J. M., Crabtree G. R., Rudikoff S., Pumphery J., Robb R. J., Kronke M., Svetlik P. B., Peffer N. J., Waldmann T. A. and Greene W. C. (1984) Molecular cloning and expression of cDNAs for the human interleukin-2 receptor. *Nature* 311, 626–631.

Malek T. R., Robb R. J. and Shevach E. M. (1983) Identification and initial characterization of a rat monoclonal antibody reactive with the murine interleukin 2 receptor–ligand complex. *Proc. natn. Acad. Sci. U.S.A.* 80, 5694–5698.

Matrisian L. M., Bowden G. T., Krieg P., Fürstenberg G., Briand J. P., Leroy P. and Breathnach R. (1986) The mRNA coding for the secreted protease transin is expressed more abundantly in malignant than in benign tumor. *Proc. natn. Acad. Sci. U.S.A.* 83, 9413.

Merrifield R. B. (1963) Solid phase synthesis. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* 85, 2149–2145.

Minami Y., Kono T., Miyazaki T. and Taniguchi T. (1993) The IL-2 receptor complex: its structure, function and target genes. *A. Rev. Immun.* 11, 245–267.

Moreau J.-L., Nabholz M., Diamanstein T., Malek T., Shevach E. and Thèze J. (1987) Monoclonal antibodies identify three epitope clusters on the mouse p55 subunit of interleukin 2 receptor: relationship to the interleukin 2-binding site. *Eur. J. Immun.* 17, 929–935.

Nakamura Y., Russel S. M., Mess S. A., Friedmann M., Erdos M., François C., Jacques Y., Adelstein S. and Leonard W. J. (1994) Heterodimerization of the IL-2 receptor β-and γ-chain cytoplasmic domains is required for signalling. *Nature* 369, 330–333.

Pitton C., Rebollo A., Van Snick J., Thèze J. and Garcia A. (1993) High-affinity and intermediate-affinity forms of the human interleukin 2 receptor, expressed in an interleukin 9-dependent murine T cell line, deliver proliferative signals via differences in their transduction pathways. *Cytokine* 5, 362–371.

Rebollo A., De Groote D., Baudrihay M., Theze J. and Jankovic D. L. (1992) Immunochemical characterization of antigenic domains of human IL-2: spatially distinct epitopes are associated with binding to the p55 and p70 subunits of IL-2 receptor. *Molec. Immun.* 29, 119–130.

Robb R. J., Kutny R. M., Panico M., Morris H. R. and Chowdhry V. (1984) Amino acid sequence and post-translational modification of human interleukin-2. *Proc. natn. Acad. Sci. U.S.A.* 81, 6486–6490.

Sauvé K., Nachman M., Spence C., Bailon P., Campbell E., Tsien W.-H., Kondas J. A., Hakimi J. and Ju G. (1991) Localization in human interleukin 2 of the binding site to the α chain (p55) of the interleukin 2 receptor. *Proc. natn. Acad. Sci. U.S.A.* 88, 4636–4640.

Severinson E., Naito T., Tokumoto H., Fukushima D., Hirano A., Hama K. and Honjo T. (1987) Interleukin-4 (IgG1 inducing factor): a multifunctional lymphokine acting also on T cells. *Eur. J. Immun.* 17, 67–72.

Smith K. A. (1988) Interleukin-2: inception, impact, and implications. *Science* 240, 1169–1176.

Takeshita T., Asao H., Ohtani K., Ishii N., Kumaki S., Tanaka N., Munakata H., Nakamura M. and Sugamura K. (1992) Cloning of the γ chain of the human IL-2 receptor. *Science* 257, 379–382.

Taniguchi T., Matsui H., Fujita T., Takaoka C., Kashima N., Yoshimoto R. and Hamuro J. (1983) Structure and

expression of a cloned cDNA for human interleukin-2. *Nature* **302**, 305–310.

Taniguchi T. and Minami Y. (1993) The IL-2/IL-2 receptor system: a current overview. *Cell* **73**, 5–8.

Teshigawara K., Wang H. M., Kato K. and Smith K. A. (1987) Interleukin 2 high-affinity receptor expression requires two distinct binding proteins. *J. exp. Med.* **165**, 223–238.

Thèze J. (1994) Cytokine receptors: a combinative family of molecules. *Eur. Cytokine Netw.* **5**, 353–368.

Tsudo M., Kitamura F. and Miyasaka M. (1989) Characterization of the interleukin 2 receptor β chain using three distinct monoclonal antibodies. *Proc. natn. Acad. Sci. U.S.A.* **86**, 1982–1986.

Uchiyama T., Broder S. and Waldmann T. (1981) A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. *J. Immun.* **126**, 1293–1297.

Uyttenhove C., Simpson R. J. and Snick J. V. (1988) Functional and structural characterization of p40, a mouse glycoprotein with T-cell growth factor activity. *Proc. natn. Acad. Sci. U.S.A.* **85**, 6934–6938.

Yodoi J., Teshigawara K., Nikaido T., Fukui K., Noma T., Honjo T., Takigawa M., Sasaki M., Ninato N., Tsudo M., Uchiyama T. and Maeda M. (1985) TCGF (IL2) receptor on a natural killer-like cell line (YT cells). *J. Immun.* **134**, 1623–1630.

Zurawski S. M., Vega F. Jr, Doyle E. L., Huyge B., Flaherty K., McKay D. B. and Zurawski G. (1993) Definition and spacial location of mouse interleukin-2 residues that interact with its heterotrimeric receptor. *Eur. molec. Biol. Org. J.* **12**, 5113–5119.

Zurawski S. M. and Zurawski G. (1992) Receptor antagonist and selective agonist derivates of mouse interleukin-2. *Eur. molec. Biol. Org. J.* **11**, 3905–3910.

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Biological and receptor-binding activities of human interleukin-2 mutated at residues 20Asp, 125Cys or 127Ser

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ABSTRACT. We have used site-directed mutagenesis to analyse structure-function relationships of the human Interleukin-2 molecule. The mutations introduced targetted residue 20Asp, within the N-terminal A helix, as well as residues 125Cys and 127Ser in the C-terminal D helix. The results presented here demonstrate that destabilizing the C-terminus α helix through introduction of Pro residues in either positions 125 or 127 reduced the biological activity of IL-2 by a factor of 10 that was correlated with a decreased ability to bind the receptor. A number of substitutions in position 20 have an even more drastic effect on biological activity and receptor binding. However, specific substitutions such as 20Asn and 20Leu displayed a differential effect on human or mouse IL-2 receptors. Furthermore, 20Leu IL-2 was found to behave as a partial antagonist of natural IL-2 when tested on murine cells.

Key words : Interleukin-2, aminoacid substitution, biological activity, receptor binding

INTRODUCTION

Interleukin-2 is one of the major cytokines regulating the immune system. Its primary biological activity consists in promoting the differentiation and clonal expansion of antigen-activated T lymphocytes. In addition, IL-2, among other activities, induces natural killer cells to become cytotoxic against a number of tumor cells or virus-infected cells. IL-2 mediates its biological activity by interacting with a specific receptor expressed at the surface of responsive cells [1]. This receptor is comprised of three IL-2 binding subunits, the IL-2Rα chain (p55), the IL-2Rβ chain (p75) and the IL-2Rγ chain [2, 3]. The tertiary structure of interleukin-2 is comprised of 4 α helices whose integrity is essential for the binding of IL-2 to its receptor and subsequent biological activity [4 - 6]. Several laboratories have undertaken the construction of mutated IL-2 proteins, on the rational that such variants have potential to behave as competitive inhibitors of natural IL-2, by preventing binding or signal transduction through the high affinity IL-2R [7-10]. However, most of the reported data concerning binding or biological activity of mutated human IL-2 have used heterologous systems (murine cell lines) as a read out for function of human IL-2 muteins. As it is known that species-specificity of IL-2/IL-2R interactions are dependent upon the IL-2Rβ chain [11], it appears important to document the biological activities of genetically engineered new molecules in an homologous system. We report here on 2 series of human IL-2 muteins targetting the carboxy-terminus of IL-2 and the N-terminal helix respectively (Fig. 1). The choice of residue substitution was based upon different strategies in the A and D helix. The main approach regarding the C-terminal helix introduced single or double Proline residues as it is known that Pro imposes a constraint on the peptide bound which is detrimental for α helix conformation. Changes in the A helix, in contrast, were conservative of the secondary structure but were aimed at substituting the presumably important side chain of Asp20. These muteins were analyzed for biological activity and receptor binding with 4 types of cells expressing receptors of different origin and combinations of the 3 subunits.

MATERIALS AND METHODS

In vitro mutagenesis, purification and biochemical characterization of IL-2 muteins. The procedure for generating IL-2 mutants has been described [12], and was based on the use of a single stranded bacteriophage containing the wild-type IL-2 coding sequence, which is grown in *dut-, ung- E. Coli* strain to obtain Uracil-containing DNA. Synthesis of the complementary strand was achieved in vitro with DNA polymerase primed with the appropriate

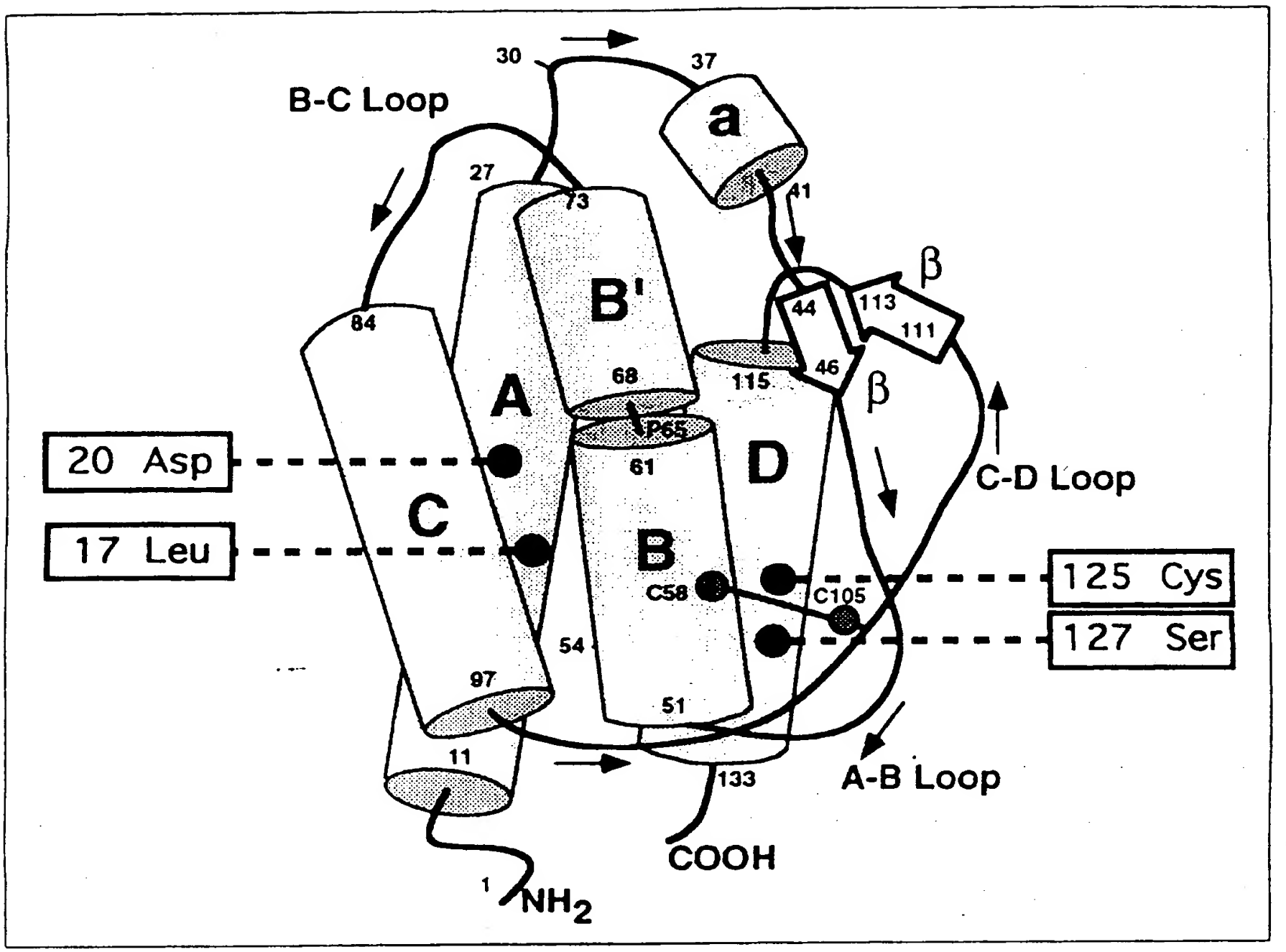

Figure 1
Sites for residue mutations in human IL-2
The human IL-2 model is adapted from reference 5. The specific residues targetted by mutations used in this paper are boxed.

mutagenic oligonucleotide. The mutated phage was then grown in wild type bacterial strain, the IL-2 insert recovered and ligated into the expression vector which was used to transform *E. Coli*. All mutations were confirmed by DNA sequencing. Purification of the mutated IL-2 molecule was achieved by preparing inclusion bodies, solubilisation and S-200 chromatography. Purity of the prepared proteins was assessed by SDS-PAGE analysis and coomassie blue staining as shown in figure 2A. In addition, a conventional western blot procedure was used (figure 2B) to show that all IL-2 muteins did react with a monoclonal antibody (19B11) that was raised against human recombinant IL-2 [13], and kindly provided by Dr Donat De Groote (Medgenix, Belgium).

Biological activity of IL-2 muteins on cells expressing different IL-2R complexes. The ability of IL-2 muteins to promote proliferation of murine CTLL-2 cells (mIL-2Rαβγ), or preactivated human T cell blasts (hIL-2Rαβγ) was assayed in 2 - 3 day cultures in microtiter plates, and DNA synthesis measured by ^{3}H-thymidine incorporation. To assess bioactivity on cells which only express the intermediate affinity βγ IL-2 receptor, the MLA-E7T subclone of the gibbon ape MLA-144 leukemia cell line was used [14]. These cells express IL-2Rβ and γ chain in the absence of α chain [15]. The gibbon IL-2Rβ chain is highly homologous to the human molecule, with only 4 minor amino acid substitutions in the extracellular domain, and no change in the SHY region which interacts with IL-2 [personal observations and ref. 16, 17]. MLA-E7T cells undergo apoptosis when cultured in the presence of glucocorticoids, in a process that is prevented by IL-2 [14 and Guizani *et al.*, submitted for publication]. To assess IL-2 mutants activity, MLA-E7T cells were cultured in microtiter plates in the pr sence of 1 μM dexamethasone, and increasing concentrations of IL-2. Cell survival was assessed using the MTT conversion assay.

The murine TS1β cell line was derived from IL-9 dependent TS1 cells which have been transfected to express the human IL-2Rβ chain. These cells thus express a functional intermediate affinity IL-2R which comprises the human IL-2Rβ chain associated to the murine IL-2Rγ chain [18].

Competitive receptor binding analysis. Binding of IL-2 muteins to the IL-2R was then investigated by their ability to compete with ^{125}I-labeled IL-2 (wild type sequence) purchased from Amersham Co. The cell lines used in these experiments include the murine IL-2 dependent CTLL-2 line expressing high affinity IL-2R, and YT cells, a human line derived from a natural killer cell leukemia which express IL-2Rβγ only [15, 19].

Binding studies were performed essentially as previously described [20]. Cell aliquots (1 to 2 x 10^6 cells in binding medium) are incubated with a constant amount of ^{125}I IL-2 for one hour at 37°C in the presence of increasing concentrations of wild type or mutant IL-2. Non specific binding is measured in the presence of a 100-fold concentration of unlabeled wild type IL-2. Bound IL-2 and free ligand are then separated by centrifuging the mixture over a phtalate oil cushion. The radioactivity of both supernatant (free ligand) and cell pellet (bound IL-2) is then measured in a γ scintillation counter and data are submitted to scatchard plot analysis.

RESULTS

Analysis of 125/127Proline IL-2 mutants. Human IL-2 molecules substituted with proline residues at position 125 or/and 127 were generated to assess the function of the C-terminal α helix in IL-2 biological activity and receptor binding. Introduction of Pro residues in either position 125 or 127 resulted in a significant loss of activity when assayed on the high-affinity receptor-expressing CTLL-2 cells or human T cell blasts (fig 3, A and B). Residual activity was however observed (12 and 6 % for 125Pro and 127 Pro IL-2 respectively), whereas the double substitution only left 0.5 - 0.7% activity (table 1). Results obtained when these mutants were assayed on TS1β (fig 3D) or MLAE7T cells (fig 3C) showed the expected one log shift in EC50, since these cells express a βγ receptor with a ~10 fold lower

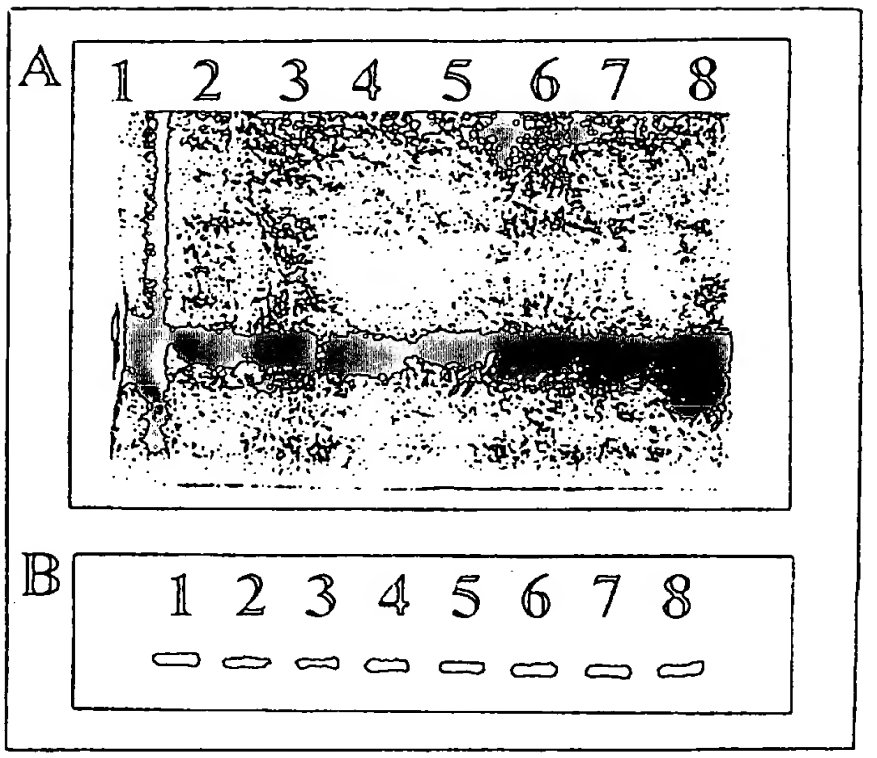

Figure 2
Purity and Immunoreactivity of IL-2 muteins
A) IL-2 proteins (5 µg) were separated by SDS-PAGE and stained with coomassie blue. B) 50 ng of the same preparations were also separated by SDS-PAGE and electroblotted onto nitrocellulose for western blot analysis. Following incubation with the 19B11 anti-IL-2 antibody and goat anti-mouse IgG coupled to peroxydase, the membrane was developped using Enhanced chemoluminescence (ECL). IL-2 muteins are identified as follows : 1, Wild type IL-2; 2, 125Pro; 3, 127Pro; 4, 125/127Pro; 5, 20Asn; 6, 20Lys; 7, 20Arg; 8, 20Leu.

Table 1
Biological activities of IL-2 muteins on different cells

Cells IL-2R expressed	CTLL-2 mαβγ		PHA blasts hαβγ		MLA-E7T "h"αβγ		TS1β hβ, mγ	
	U/mg	%WT	U/mg	%WT	U/mg	%WT	U/mg	%WT
wild type	1.3×10^7	100	4.2×10^6	100	2.4×10^6	100	4.3×10^4	100
125PRO	1.5×10^6	11.8	5×10^5	11.9	7×10^5	28.1	3.9×10^3	9
127PRO	6.4×10^5	5	2.5×10^5	5.9	2.7×10^5	11.2	3.1×10^2	0.7
125/127PRO	6.6×10^4	0.5	3.2×10^4	0.7	5.5×10^4	1.5	---	--
20ASN	1.8×10^6	14	8×10^4	1.9	1.1×10^4	0.5	6.2×10^2	1.4
20LYS	1.4×10^4	0.1	4×10^3	0.1	1.2×10^3	0.5	---	--
20LEU	---	--	5.6×10^2	0.01	---	--	---	--
20ARG	2.1×10^2	--	---	--	---	--	---	--

The biological activity was measured in proliferation assays and expressed as units/mg of protein (U/mg)

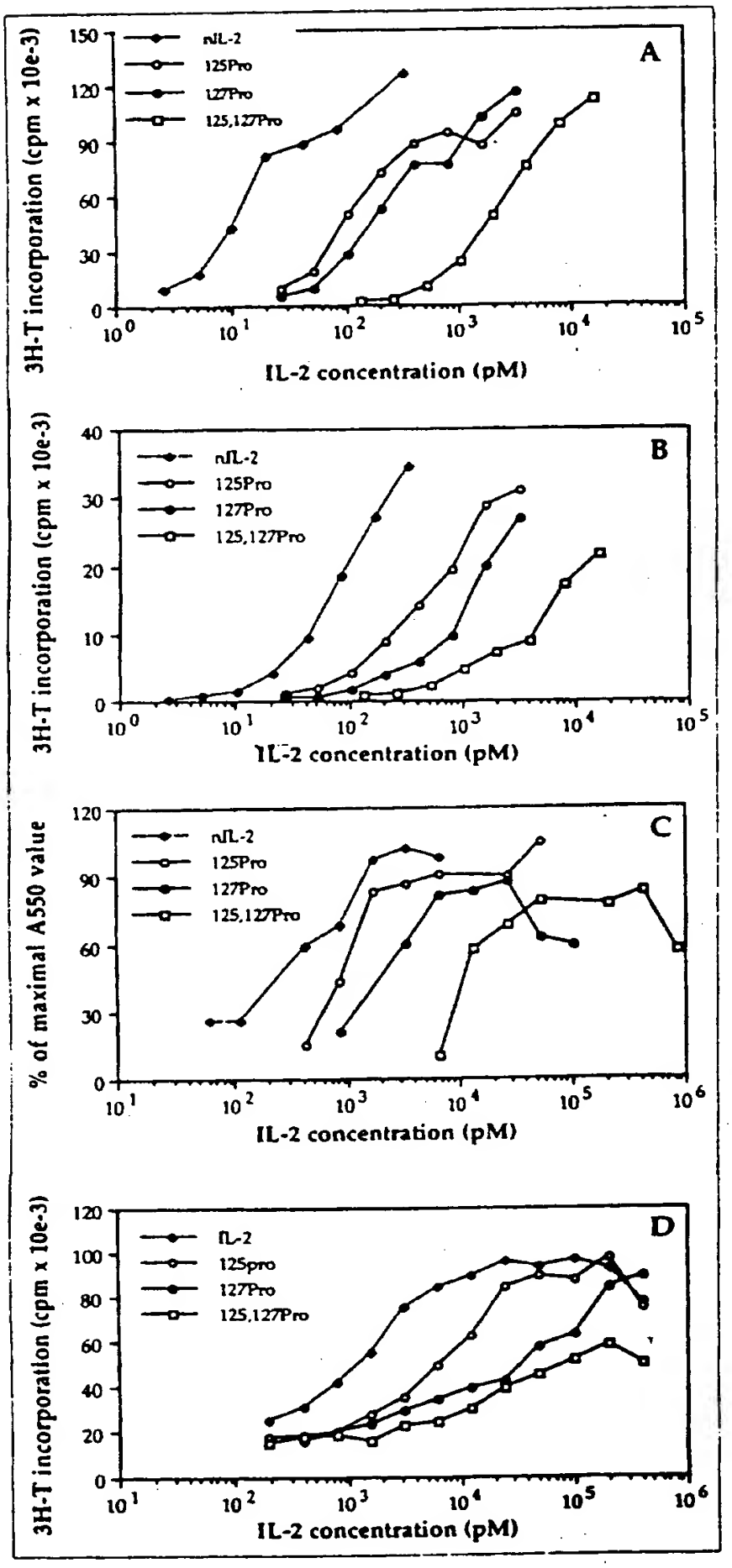

Figure 3

Biological activities of 125/127 Pro IL-2 muteins

The biological activity of 125/127 Pro IL-2 muteins was assayed on CTLL-2 cells (panel A), human T cell blasts (panel B), MLAE7T cells (panel C) and TS1β cells (panel D). IL-2 was added to all cells, in increasing concentrations in microtiter plates. In A, B, and D, cell proliferation was measured by ^{3}H-thymidine incorporation. In C, MLAE7T survival was evaluated by the MTT dye conversion assay, in the presence of 1 μM Dex and the indicated concentrations of IL-2.

affinity. It should however be noted that all three mutants had a proportionnally better activity in the MLAE7T assay than in the T cell blast assay, suggesting that altering the C-terminal helix conformation may indirectly affect intractions with the IL-2Rα chain. However most of the loss in activity probably results from hampered interaction with the γ chain which interacts with IL-2 at the level of residue 126Gln.

The ability of IL-2 muteins to compete with wild-type IL-2 for binding to the IL-2 receptor was then analysed using murine CTLL-2 or human YT cells (fig 4, A and B respectively). On each cell line, the experiments were performed under predetermined conditions which allow 90% saturation of the receptors by ^{125}I-IL-2. All three IL-2 mutants demonstrated a reduced binding to IL-2 receptors of either high (CTLL-2) or intermediate-affinity (YT). This correlates with the reduction in bioactivity. Comparison of the results obtained with these two cell lines suggests that the double mutant 125/127 Pro-Pro has the poorest interaction with the IL-2Rα chain

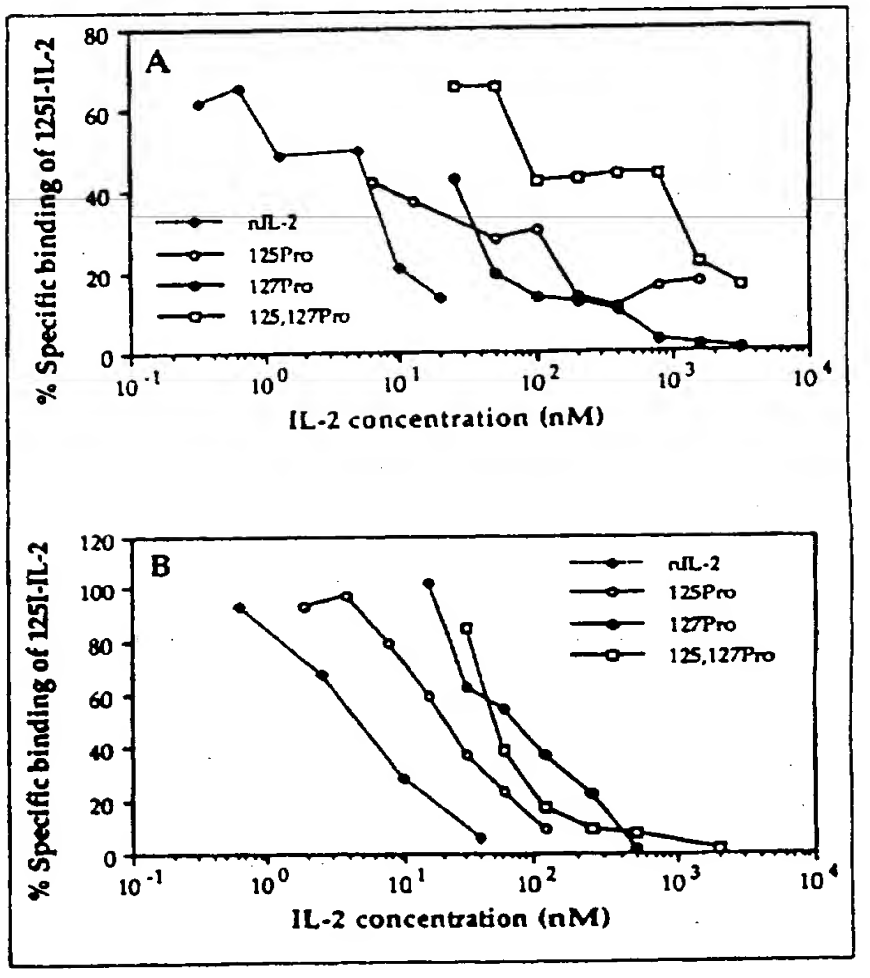

Figure 4

Competitive IL-2R binding of 125/127 Pro IL-2 muteins

Interactions of D helix IL-2 mutants with IL-2 receptors were analyzed on CTLL-2 cells (A) or YT cells (B). CTLL-2 cells were incubated with 200 pM , and YT cells with 600 pM iodinated IL-2 in the presence of increasing concentrations of either wild-type IL-2, or IL-2 muteins. Specific binding was calculated by subtracting background binding obtained in the presence of a 100 fold excess of wild-type IL-2. Data are expressed as percentage of maximum specific binding obtained in the absence of inhibitor.

since its binding to the YT cell IL-2R is similar to that of 125ProIL-2 or 127ProIL-2, whereas it is much lower in the CTLL-2 competitive binding assay.

These results thus confirm and extend previous studies indicating that the critical feature of IL-2 C-terminus is its α helical conformation.

Analysis of IL-2 mutants in position 20. Mutation of Aspartic acid in position 20 of the human IL-2 molecule appears to be very detrimental to biological activity. Indeed, regardless of the assay system, very little activity is left when Asp 20 was mutated into either uncharged Leu or positively charged Arg or Lys, thus indicating that the negative charge of Asp may be involved in IL-2/IL-2R interactions. However substitution with Asn left some detectable bioactivity on both human and murine cells. The β chain of IL-2R is thought to confer some species specificity to the molecule, and it has recently been shown that Asp 20 of human IL-2, which stands as the equivallent of Asp34 in murine IL-2 is directly involved in the binding to IL-2Rβ. In keeping with these observations, it is of interest to note that several mutants have slightly different activity on human PHA blasts and on murine CTLL-2 cells (fig. 5). In particular, in agreement with work by others [21, 22] substitution of 20Asp with Asn left an approximate 15% biological activity when assayed on CTLL-2, but only 1.9% on human T cell blasts. The reverse is true for 20Leu IL-2 which is completely inactive on CTLL-2 cells and can yet induce proliferation of human PHA blasts when used at high concentration.

In keeping with the bioactivity data, competitive binding analysis (fig 6) showed that 20Asn IL-2 was slightly more efficient at displacing ^{125}I-IL-2 from the murine receptor than from the human receptor. The other muteins, 20Leu, 20Lys and 20Arg displayed a roughly equivallent binding ability on both types of receptors. These results thus demonstrate that, as initially hypothesized, some of the mutations in position 20 might affect differentially IL-2 binding to the murine or human IL-2R β chain. Since both murine and human IL-2Rβ chains contain the restricted SHY sequence which mediates interaction with IL-2 [16], it is likely that adjacent residues which differ slightly between the two sequences, may contribute to the binding site.

20Leu IL-2 behaves as a partial antagonist for murine CTLL-2 cells. The experiment described above contain an apparent paradox regarding the biological activity and receptor binding of 20Leu IL-2. 20Leu IL-2 can induce proliferation of human T cell blasts, and although completely inactive on CTLL-2 cells, does not perform worse than the other 20-position muteins in competitive binding assays. Mix-in experiments were thus undertaken to assess the possibility that 20Leu IL-2 might behave as an inhibitor of wild-typ IL-2 on CTLL-2 cells. For this purpose, CTLL-2 cells were cultured in the presence of a suboptimal

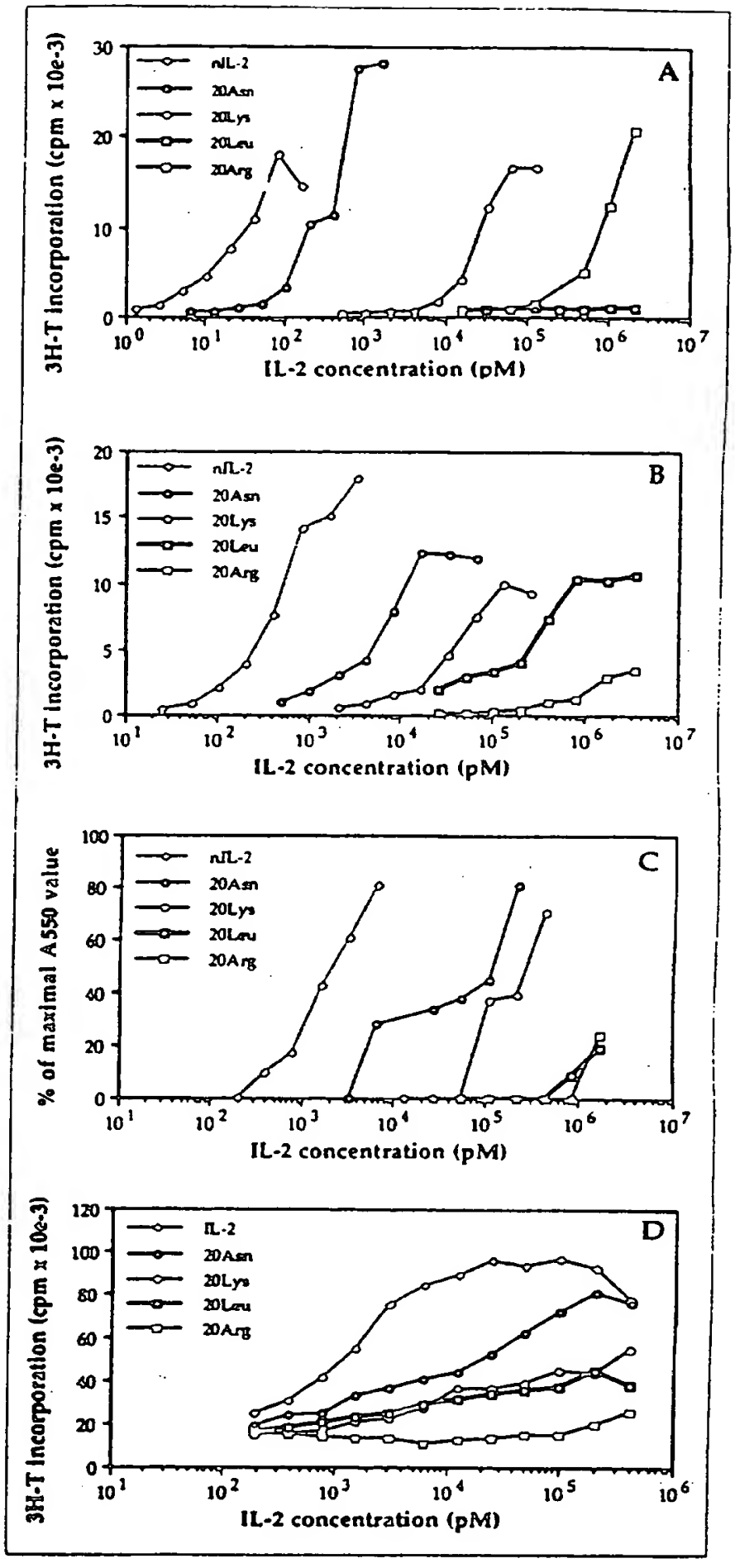

Figure 5
Biological activities of IL-2 proteins mutated in position 20
The biological activity of IL-2 proteins mutated in position 20 was assayed on CTLL-2 cells (panel A), human T cell blasts (panel B), MLAE7T cells (panel C) and TS1β cells (panel D). Cell proliferation or survival was measured as in fig. 2.

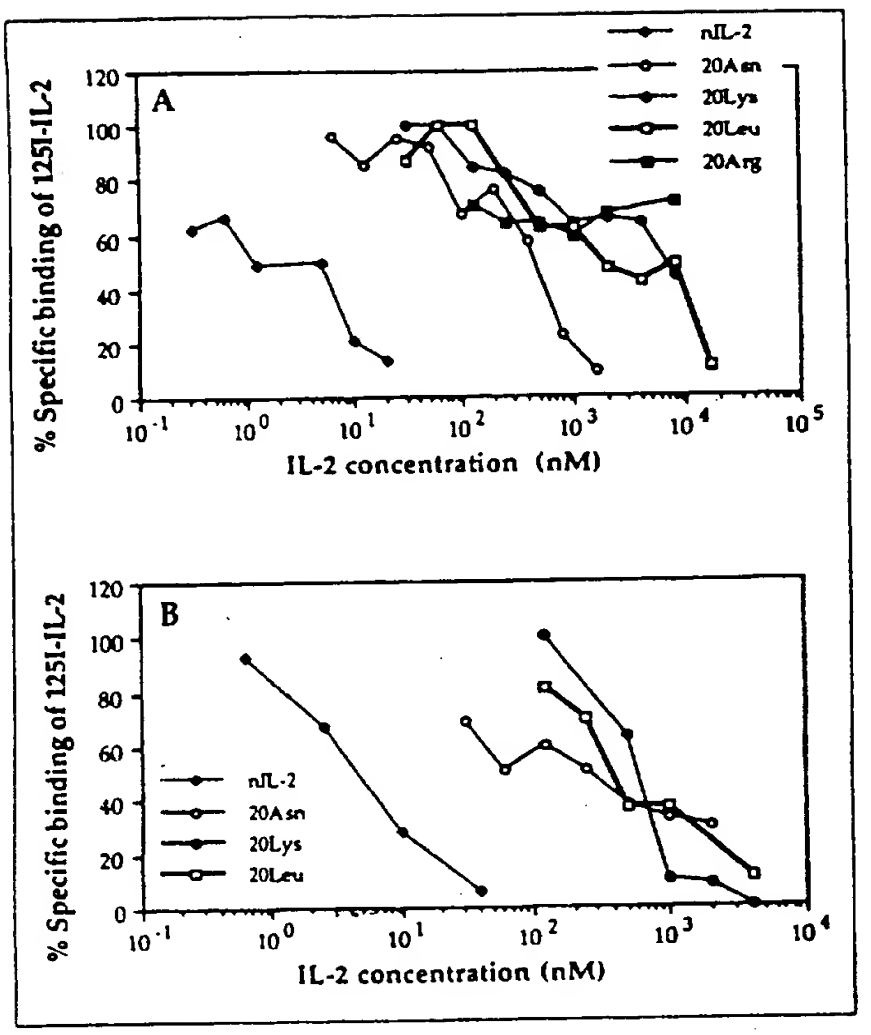

Figure 6
Competitive IL-2R binding of IL-2 proteins mutated in position 20
Binding of position-20 IL-2 muteins was assessed on CTLL-2 cells (A) or YT cells (B) exactly as in figure 3.

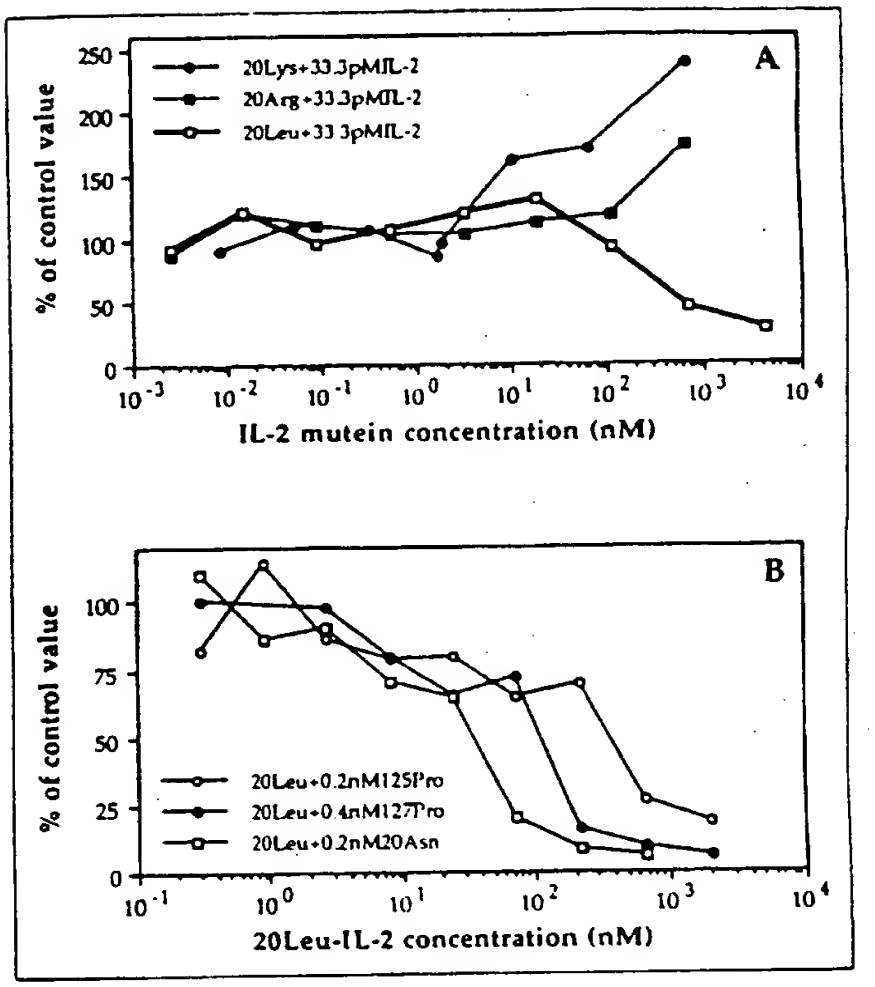

Figure 7
20Leu IL-2 behaves as a partial antagonist
(A) CTLL-2 cells were cultured in the presence of 33pM wild-type IL-2 and increasing concentrations of 20Lys, 20Arg or 20Leu IL-2 muteins. (B) Titration of 20Leu IL-2 inhibitory effect assessed against 125Pro, 127Pro and 20Asn muteins. ^{3}H-thymidine incorporation was measured in a 3-day assay, and data are expressed as percentage of the maximum incorporation obtained in the absence of inhibitor.

concentration of wild type IL-2 (33pM) and increasing concentration of muteins. As shown in fig 7A, an additive and dose dependent effect was observed with 20Lys or 20Arg IL-2, whereas addition of 20Leu IL-2 inhibited CTLL-2 cell proliferation. 50% inhibition was obtained at a concentration of 20Leu IL-2 of around 500nM, that is a 15,000 fold molar excess over wild-type IL-2. In order to sensitize the assay, a series of experiments was performed using appropriate concentrations of 125Pro IL-2 (0.2nM), 127Pro IL-2(0.4nM) or 20Asn IL-2(0.2nM) to drive the basal CTLL-2 proliferation (fig 7B). These experiments confirmed the inhibitory activity of 20Leu IL-2, which induced 50% inhibition when used in molar excess of 2000, 250 and 150 against 125Pro IL-2, 127Pro IL-2 and 20Asn IL-2 respectively.

DISCUSSION

Detailed studies of murine IL-2 have clearly shown that integrity of the first α helix (A region) was important for IL-2Rβ binding and biological function, whereas mutations which only affect binding to the IL-2Rα chain had little functional consequences [21, 22]. Previous studies with the human IL-2 molecule, have confirmed the critical importance of amino-acid residues within the N-terminal α helix (region A) and C-terminal helix (region D) [13, 23 - 24]. Taken together, the published reports lead to the currently accepted model of IL-2/IL-2R interactions, whereby IL-2 contacts all three IL-2R chains simultaneously: the negatively charged side chain of aspartic acid in position 34 of murine IL-2 (corresponding to position 20 in the human molecule) is essential for binding to the IL-2Rβ chain, and glutamine 141 in murine IL-2 (126 in human IL-2) is involved in contacting the γ chain [7 - 10]. Interactions with the α chain appear to depend upon the overall structure of the molecule and contact is thought to be mediated through a short helical segment, refered to as helix E in the initial model of Brandhuber *et al.* [4] located within the loop joining the A and B α helices in the current model [5, 21]

The experiments reported above confirm and extend previously reported informations regarding structure-function relationships of the human IL-2 molecule [6 - 8 and 21 - 24]. Clearly of importance, is the α

helical conformation of IL-2 carboxy terminus. Indeed introducing proline residues in this region affects binding to IL-2 receptors and biological function. Reduction of the 125/127 Pro D-helix mutants interaction with the IL-2Rγ chain, which primarily depends upon 126Gln is likely to be due to disruption of the α helix, although it can not be excluded that the direct proximity to 126Gln of these substitutions may play a role. On the other hand, bioactivity of 125/127Pro mutants on high-affinity receptor-expressing cells is also reduced, suggesting a possible indirect effect of D helix on interactions with the IL-2Rα chain.

In this report, we also confirm the critical role of 20Asp, which is thought to mediate contact with the IL-2Rβ chain. Mutating 20Asp into various amino acids is, generally speaking very detrimental to biological function, although analysis of the residual activity allowed us to identify some species specificity of IL-2. Indeed, whereas the 20Asn mutein was fairly active on murine cells, it was not, when assayed on human T-cell blasts. Furthermore, the reverse was true of the 20Leu mutation, which was demonstrated to specifically inhibit the effect of wild type IL-2 on murine cells, eventhough high concentrations of the mutein were required. Understanding the exact molecular basis for the species specificity of the 20Leu mutein will require additional experiments. With the available informations, it is however possible to speculate that, confronted to a murine receptor, 20Leu IL-2 will bind to the γ chain only, and not interact with the β chain, a situation that would not trigger proliferative signalling. On human cells however, residual binding to the β chain, together with normal binding to γ should result in some level of biological activity.

Finally, although the experiments reported here only addressed the function of the 20Asp residue and of conformation of the D helix, it should be noted that a number of other residues, important for IL-2 function include 62Glu [12] as well as 17Leu. During the course of these studies, we have observed, like others [23, 24], that 17Leu was critical for function and that swapping 17Leu and 20Asp yielded a completely inactive molecule (not shown). Altogether, this site-directed mutagenesis approach, which has proven quite effective in engineering an IL-4 antagonist [25], has so far failed to generate IL-2 molecules which could be of any practical use in the laboratory or in the clinic. Yet, the data presented here on the inhibitory effect of 20Leu IL-2 suggest that the design of IL-2 antagonists through directed mutagenesis, although complex, should be achievable.

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REFERENCES

1. Smith K A. 1988. Interleukin-2: inception, impact and implications . *Science* 240 :1169.

2. Takeshita T, Asao H, Ohtani K, Ishii N, Kumaki S, Tanaka N, Munakata H, Nakamura M and Sugamura K. 1992. Cloning of the γ chain of the human IL-2 receptor. *Science* 257:379.

3. Taniguchi T and Minami T. 1993. The IL-2/IL-2 receptor system: a current overview. *Cell* 73: 5.

4. Brandhuber B J, Boone T, Kenney W C and McKay D B. 1987. Three-Dimensional Structure of Interleukin-2. *Science* 23:1707.

5. Bazan J F. 1992. Unraveling the Structure of IL-2. *Science* 257:410.

6. Landgraft B, Cohen F E, Smith K A, Gadski R and Ciardelli T L. 1989. Structural Significance of the C-terminal Amphiphilic Helix of Interleukin-2. *J. Biol Chem* 264:816.

7. Zurawski S M, Imler J L and Zurawski G. 1990. Partial agonist/antagonist mouse interleukin-2 proteins indicate that a third component of the receptor complex functions in signal transduction. *EMBO J.* 9:3899.

8. Fleimming C L, Russell S J and Collins M K L. 1993. Mutation of Asp20 of human interleukin-2 reveals a dual role of the p55 α chain of the interleukin-2 receptor. *Eur. J Immunol.* 23:917.

9. Voss S D, Leary T P, Sondel P M and Robb R J. 1993. Identification of a direct interaction between interleukin 2 and the p64 interleukin 2 receptor γ chain. *Proc Natl Acad. Sci. USA* 90:2428.

10. Zurawski S M and Zurawski G. 1992. Receptor antagonist and selective agonist derivatives of mouse interleukin-2. *EMBO J.* 11:3905.

11. Chastagner P, Moreau J-L, Jacques Y, Tanaka T, Miyasaka M, Kondo M, Sugamura K and Thèze J. Lack of intermediate affinity IL-2 receptor in mice leads to a dependence on IL-2 receptor α chain for T cell growth. *Eur. J. Immunol.* in press.

12. Wang Z, Zheng Z, Sun L and Liu X. 1995. Substitutions at the Glu62 residue of human interleukin-2 differentially affect its binding to the α chain and the βγ complex of the interleukin-2 receptor. *Eur. J. Immunol.* 25:1212.

13. Moreau J-L, Bossus M., De Groote D, François C, Jacques Y, Tartar A and Thèze J. Characterization of a monoclonal antibody directed against the NH2 terminal area of interleukin-2 (IL-2) and inhibiting specifically the binding of IL-2 to IL-2 receptor β chain. *Mol. Immunol.* in press.

14. Smith, K. 1988. The Interleukin 2 receptor. *Adv. Immunol.* 42:165.

15. Guizani L, Lang P, Stancou R and Bertoglio J. 1993. Regulation of interleukin-2 and Interleukin-4 Receptor Expression in Human and Ape Lymphoid Cell lines. *Lymphokine and Cytokine Research* 12:25.

16. Imler J L, Miyajima A and Zurawski G. 1992. Identification of three adjacent amino acids of interleukin-2 receptor β chain which control the affinity and the specificity of the interaction with interleukin-2. *EMBO J.* 11:2047.

17. Kono T, Doi T, Yamada G, Hatakeyama M, Minamoto S, Tsudo M, Miyasaka M, Miyata T and Taniguchi T. 1990. Murine interleukin 2 receptor β chain: Dysregulated gene expression in lymphoma line EL-4 caused by a promoter insertion. *Proc. Natl. Acad. Sci. USA* 87:1806.

18.Pitton C, Rebollo A, Van §nick J, Thèze J and Garcia A. 1993. High-affinity and intermediate-affinity forms of the human interleukin-2 receptor, expressed in an interleukin 9-dependent murine T cell line, deliver proliferative signals via differences in their transduction pathways. *Cytokine* 5:362.

19. Yodoi T, Teshigawara K, Nikaido T, Fukui K, Noma T, Honjo T, Takigawa M, Sasaki M, Minato N, Tsudo M, Uchiyama T and Maeda M. 1985. TCGF (IL2)-receptor inducing factor: Regulation of IL2 receptor on a natural killer-like cell line. *J. Immunol.* 134:1623.

20 Bertoglio J, Dosda J, Stancou R, Wollman E and Fradelizi D. 1989. Expression and regulation of interleukin-1 mRNA and interleukin-1 receptors in human B-cell lines. *J. Mol. Cell. Immunol.* 4:139.

21. Zurawski S M and Zurawski G.1989. Mouse interleukin-2 structure - function studies : substitutions in the first α-helix can specifically inactivate p70 receptor binding and mutations in the fifth α-helix can specifically inactivate p55 receptor binding. *EMBO J.* 8:2583.

22. Zurawski S M and Zurawski G. 1988. Identification of three critical regions within mouse interleukin 2 by fine structural deletion analysis. *EMBO J.* 7:1061.

23. Collins L, Tsien W H, Seals C, Hakimi J, Weber D, Bailon P, Hoskings J, Greene W C, Toome V and Ju G.1988. Identification of specific residues of human interleukin 2 receptor binding to the 70-kDa subunit (p70) of the interleukin 2 receptor. *Proc Natl. Acad Sci. USA* 85:7709.

24. Ju G, Collins L, Kaffka K L, Tsien W H, Chizzonite R, Crowl R, Bhatt R and Kilian P L. 1987. Structure-Function Analysis of Human Interleukin-2. *J. Biol. Chem.* 262:5723.

25. Kruse N, Tony HP and Sebald W. 1992. Conversion of human Interleukin-4 into a high affinity antagonist by a single amino acid replacement. *EMBO J.* 11:3237.

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codon modifications:									t	c		c			c		g	g	
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CTC	GAG	CAC	CTG	CTG	CTG	GAT	TTG	CAG	ATG	ATC	CTG	AAC	GGT	ATC	AAC	AAT	TAC	AAG	AAC
XhoI																			
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g		g		c	t		g	c	c		c			g			c		
CCG	AAA	CTG	ACG	CGT	ATG	CTG	ACC	TTC	AAG	TTC	TAC	ATG	CCG	AAG	AAG	GCC	ACC	GAA	CTG
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						60												70	
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aaa	cat	ctt	cag	tgt	cta	gaa	gaa	gaa	ctc	aaa	cct	ctg	gag	gaa	gtg	cta	aat	cta	gct
	c	g			g			g		g				t	g	c	c	g	
AAA	CAC	CTG	GAG	TGT	CTA	GAA	GAA	GAA	CTG	AAA	CCG	CTG	GAG	GAA	GTT	CTG	AAC	CTG	GCT
					XbaI														

(57) Abstract

A mutant IL-2 molecule capable of binding an IL-2 receptor-bearing cell, having a deletion of one to five amino acid residues of IL-2, the deletion resulting in active IL-2 molecules that have increased resistance to proteolysis.

* See back of page

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IL-2 DELETION MUTANTS

Background of the Invention

This invention relates to the use of recombinant DNA techniques to make mutant interleukin-2 (IL-2, molecules and chimeric IL-2/toxin molecules.

Il-2 is a protein secreted by human T-lymphocytes which is capable of binding to IL-2 receptors on activated T-lymphocytes and effecting T-lymphocyte proliferation. IL-2 has been shown to be a therapeutic immunostimulant in humans (Rosenberg, 1988, Immunology Today 9:2: 58-62), and IL-2 or a specific binding portion thereof can be coupled to the enzymatically active portion of diphtheria toxin to form a hybrid molecule with a number of therapeutic applications (Murphy U.S. Patent No. 4,675,382, hereby incorporated by reference). IL-2/diphtheria toxin hybrid proteins of Murphy '382, which were made using recombinant DNA techniques, have been shown to inhibit rejection of transplanted organs (Pankewycz et al., Transplantation 47:318-322 (1989)), and are also potential therapeutic agents in the treatment of certain cancers and autoimmune diseases in which the IL-2 receptor plays a role.

IL-2 encoding DNA sequences are reported in a number of publications, and in addition, a modified IL-2-encoding gene, in which a cysteine codon is changed to enhance stability, is described in U.S. Pat. No. 4,518,584, hereby incorporated by reference. U.S.S.N. 834,900, filed Feb. 28, 1986, hereby incorporated by reference, describes a synthetic IL-2-encoding DNA

sequence that differs from the natural IL-2 encoding DNA in that it contains more prokaryotic preferred translation codons than the naturally occurring sequence.

Amino acid deletions or substitutions have been made in the IL-2 amino acid sequence (European Pat. Appln. Nos. 86114468.1 and 87101839.6, U.S. Pat. No. 4,604,377). Although the DNA and amino acid sequences of IL-2 and its crystal structure are known (Brandhuber et al., 1987, Science 238, 1707), there is little data available that allows accurate prediction of the regions of IL-2 that are responsible for biological activity or are sensitive to proteolytic breakdown; e.g., a single substitution of the cysteine residue at position 125 of the IL-2 amino acid sequence with a serine results in increased stability of the molecule (U.S. Patent No. 4,604,377); a substitution of the tryptophan residue at position 121 inactivates the molecule; deletion of amino acid residues 100-104 decreases the biological activity by two oders of magnitude; and deletion of amino acid residues 124-126 renders the molecule inactive (Collins et al., 1988, Proc. Nat. Aca. Sci. 85: 7709; Cohen et al., 1986, Science 234:349).

Summary of the Invention

The present invention provides IL-2 mutant polypeptides that bear a deletion of one to five amino acids, yet retain the ability to bind to IL-2 receptor-bearing cells. It is known that lysine 76 is a proteolytic site in the IL-2 molecule (Cohen et al., 1986, Science 234:349). These mutants either delete this proteolytic site completely, or alter the structure of that area in an effort to reduce proteolysis. The IL-2 mutants can be used as immunostimulants or, when coupled to a toxin to form a hybrid IL2-toxin molecule,

can be used to treat immune and other disorders characterized by the presence of the IL-2 receptor.

The invention thus generally features eight new mutant IL-2 polypeptides capable of binding to the IL-2 receptor; the IL-2 polypeptides have deletions of one or more amino acid residues, as follows: 74; 74-78; 75-77; 76-78; 76-79; 75, 78; and 79 (according to the numbering convention of the Figure, taken from Williams et al., Nucleic Acids Res., vol. 16, no. 22 (1988).

In some preferred embodiments, the mutant IL-2 polypeptide may be part of a fusion protein consisting of a toxin portion (e.g., derived from diphtheria toxin) covalently linked, preferably through a peptide bond at its carboxy terminal end, to the mutant IL-2 polypeptide. The diphtheria toxin portion is large enough to exhibit cytotoxic activity and small enough to fail to exhibit generalized eukaryotic cell binding.

Preferably, the DNA sequence encoding the IL-2 polypeptide contains nucleotide substitutions designed to maximize gene expression in the cells used for expression; i.e., where prokaryotic cells such as E. coli are used, preferred prokaryotic codons are substituted for some of the natural codons (this has been done in the sequence shown in the Figure).

The hybrid molecules of the invention are useful for treating diseases in which the IL-2 receptor plays a role, e.g., IL-2 receptor positive malignancies, allergic reactions, and systemic lupus erythmatosis (SLE), or to prevent an immune response by IL-2 receptor bearing T cells that occurs in graft rejection. This targeted toxin functions by the following mechanism: the IL-2/toxin, by virtue of the IL-2 domain, binds to high affinity IL-2 receptor-bearing cells. The IL-2-toxin is internalized into endocytic vesicles by IL-2 receptor-mediated endocytosis. Acidification of the

endosome causes a conformational change in the toxin, allowing its membrane-associating domains to interact with the endocytic vesicle's membrane and facilitate translocation of the enzymatically active fragment A into the cytosol. Once delivered to the cytosol, fragment A catalyzes the ADP-ribosylation of elongation factor 2, resulting in inhibition of protein synthesis and subsequent death of the IL-2-receptor bearing cell.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawing is first described.

Drawing

The Figure is a DNA sequence, encoding IL-2, in which preferred prokaryotic translation codons are employed; the numbers correspond to the numbering referred to in this specification.

Construction of the Genes Encoding IL-2 Deletion Mutants/Toxin

Amino acids 74 through 79 are contained within the Xba1/Not1 fragment of the synthetic IL-2 gene (see Figure). For each of the eight deletion mutants, an Xba1/Not1 fragment with a deletion of DNA encoding between one and five amino acids is synthesized using an automated DNA synthesizer according to conventional techniques. The DNA sequences of the oligonucleotides are shown in Table I.

Each Xba1/Not1 fragment is synthesized as two complementary strands with a 1/2 Xba1 site at the 5' end and a 1/2 Not1 site at the 3' end. The synthetic DNA's are gel purified on a denaturing polyacrylamide-urea gel and complementary strands are annealed according to conventional methods. The annealed DNA's are ligated

into the expression plasmid, pDW15 (Williams et al., 1987, Prot. Engineering 1:493), which contains the synthetic IL-2 gene shown in the Figure. Ligation reactions are transformed into a suitable E. coli host according to conventional techniques.

Transformants are screened by restriction digest analysis of minilysate DNA using the restriction enzyme DdeI. The DdeI restriction digest profile of the IL-2 mutants differs from that of non-deleted IL-2 due to elimination of a DdeI site within the XbaI/NotI fragment of the deletion mutants. The DNA sequence of the IL-2 deletion mutants are confirmed by the dideoxy method of Sanger et al. (1977, Proc. Nat. Acad. Sci., 74:5463).

The genes encoding the IL-2/diphtheria toxin fusion proteins are constructed by standard recombinant DNA techniques, as follows. The IL-2 portion of the fusion gene is contained within the SphI/HindIII fragment of the IL-2 deletion mutant derived from pDW15. This DNA fragment is ligated to SphI/HindIII digested plasmid pABM6508 (Bishai et al., 1987, J. Bacteriol, 169:5140), which contains the diphtheria toxin-related portion of the fusion up to and including the amino acid residue Ala 486. The DNA is transformed into a suitable E. coli host and plated onto Luria broth plates plus an appropriate antibiotic for selection, according to conventional techniques. Transformants are screened by DdeI restriction digest analysis of minilysate DNA and by Western blot analy is, as follows.

Western Blot Analysis

Total bacterial cell lysates are analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970, Nature 227:680) for the production of IL-2/toxin protein. Proteins are electroblotted onto nylon

membrane and immunoblot analysis is performed according to conventional techniques. Confirmation of the expected construct is made by positive cross-reactivity to both anti-diphtheria toxin (Connaught Laboratories, Toronto, Ontario, Canada) and to a monoclonal anti-IL-2 antibody, as well as by comparison of the size of the expressed protein to known IL-2/toxin standard. Final confirmation of the construct is made by DNA sequence analysis of the IL-2//toxin gene.

Cytotoxicity assay

Referring to Table II, C91/P1 cells (a high-affinity IL2 receptor-bearing cell line) were seeded in 96-well V-bottom plates (Nunc, Roskilde, Denmark) at a concentration of 10^5 per well in 100 µl complete medium. Il-2-toxin was added at varying concentrations (10^{-12}M to 10^{-6}M) in complete medium. Cells cultured with medium alone were included as the control. Following 18 hours incubation at 37°C in a 5% Co_2 atmosphere, the plates were centrifuged for 5 minutes at 170 x g, the medium was removed and replaced with 100 µl leucine-free medium (DMEM Selectamine, Gibco) containing 2.5 µCi/ml [^{14}C]-leucine (New England Nuclear, Boston, MA). Cells were then incubated at 37° for 90 minutes and collected on glass fiber filters using a cell harvester (Skatron, Sterling, VA). Filters were washed, dried, and counted according to standard methods. All determinations were performed in pentuplicate. IC_{50} refers to the concentration of IL2 required to inhibit protein synthesis to 50% of the untreated control.

IL-2 coding sequence →	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83
(5' → 3')	T	CTA	GAA	GAA	GAA	CTG	AAA	CCG	CTG	GAG	GAA	GTT	CTG	AAC	CTG	GCT	CAG	TCT	AAA	AAC	TTC	CAC	CTG	CGG	CCG	CG
amino acid →		Leu	Glu	Glu	Glu	Leu	Lys	Pro	Leu	Glu	Glu	Val	Leu	Asn	Leu	Ala	Gln	Ser	Lys	Asn	Phe	His	Leu	Arg	Pro	
psI 133(Δ74)	T	CTA	GAA	GAA	GAA	CTG	AAA	CCG	CTG	GAG	GAA	GTT	CTG	AAC	CTG	GCA	TCT	AAA	AAC	TTC	CAC	CTG	CGG	CCG	CG	
		Leu	Glu	Glu	Glu	Leu	Lys	Pro	Leu	Glu	Glu	Val	Leu	Asn	Leu	Ala	Ser	Lys	Asn	Phe	His	Leu	Arg	Pro		
psI 134(Δ75)	T	CTA	GAA	GAA	GAA	CTG	AAA	CCG	CTG	GAG	GAA	GTT	CTG	AAC	CTG	GCA	CAG	AAA	AAC	TTC	CAC	CTG	CGG	CCG	CG	
		Leu	Glu	Glu	Glu	Leu	Lys	Pro	Leu	Glu	Glu	Val	Leu	Asn	Leu	Ala	Gln	Lys	Asn	Phe	His	Leu	Arg	Pr		
psI 136(Δ78)	T	CTA	GAA	GAA	GAA	CTG	AAA	CCG	CTG	GAG	GAA	GTT	CTG	AAC	CTG	GCA	CAG	TCT	AAA	AAC	CAC	CTG	CGG	CCG	CG	
		Leu	Glu	Glu	Glu	Leu	Lys	Pro	Leu	Glu	Glu	Val	Leu	Asn	Leu	Ala	Gln	Ser	Lys	Asn	His	Leu	Arg	Pro		
psI 137(Δ79)	T	CTA	GAA	GAA	GAA	CTG	AAA	CCG	CTG	GAG	GAA	GTT	CTG	AAC	CTG	GCA	CAG	TCT	AAA	AAC	TTC	CTG	CGG	CCG	CG	
		Leu	Glu	Glu	Glu	Leu	Lys	Pro	Leu	Glu	Glu	Val	Leu	Asn	Leu	Ala	Gln	Ser	Lys	Asn	Phe	Leu	Arg	Pro		
psI 143(Δ75-77)	T	CTA	GAA	GAA	GAA	CTG	AAA	CCG	CTG	GAG	GAA	GTT	CTG	AAC	CTG	GCA	CAG	TTC	CTG	CGG	CCG	CG				
		Leu	Glu	Glu	Glu	Leu	Lys	Pro	Leu	Glu	Glu	Val	Leu	Asn	Leu	Ala	Gln	Phe	Leu	Arg	Pro					
psI 141(Δ74-78)	T	CTA	GAA	GAA	GAA	CTG	AAA	CCG	CTG	GAG	GAA	GTT	CTG	AAC	CTG	GCA	CAC	CTG	CGG	CCG	CG					
		Leu	Glu	Glu	Glu	Leu	Lys	Pro	Leu	Glu	Glu	Val	Leu	Asn	Leu	Ala	His	Leu	Arg	Pro						
psI 150(Δ76-79)	T	CTA	GAA	GAA	GAA	CTG	AAA	CCG	CTG	GAG	GAA	GTT	CTG	AAC	CTG	GCA	CAG	TCT	CAC	CTG	CGG	CCG	CG			
		Leu	Glu	Glu	Glu	Leu	Lys	Pro	Leu	Glu	Glu	Val	Leu	Asn	Leu	Ala	Gln	Ser	His	Leu	Arg	Pro				
psI 145(Δ76-78)	T	CTA	GAA	GAA	GAA	CTG	AAA	CCG	CTG	GAG	GAA	GTT	CTG	AAC	CTG	GCA	CAG	TCT	CTG	CGG	CCG	CG				
		Leu	Glu	Glu	Glu	Leu	Lys	Pro	Leu	Glu	Glu	Val	Leu	Asn	Leu	Ala	Gln	Ser	Leu	Arg	Pro					

TABLE I

Table II

Plasmid	amino acid(s) deleted	C91/PL IC50
psI133	Φ74	$6x10^{-1}M$
psI134	Φ75	$1x10^{-10}M$
PsI136	Φ78	$5x10^{-11}M$
psI137	Φ79	$2x10^{-10}M$
psI143	Φ75-77	$2x10^{-10}M$
psI141	Φ74-78	$1x10^{-10}M$
psI145	Φ76-78	$2x10^{-10}M$
psI150	Φ76-79	$7x10^{11}M$
(psI129 control	no deletion	typically $5x10^{-11}M$)

Other Embodiments

Other embodiments are within the following claims. For example, the deletion mutant IL-2 molecules can be used alone, in addition to their use in toxic hybrids, the deletions can advantagously provide resistance to proteolysis in both contexts. In addition, toxins other than diphtheria toxin can be coupled to the mutants, e.g., the enzymatically active portion of Pseudomonas exotoxin can be used.

Claims

1. A mutant IL-2 molecule in which only amino acid residue 74 has been deleted.

2. A mutant IL-2 molecule in which only amino acid residues 74-78 have been deleted.

3. A mutant IL-2 molecule in which only amino acid residues 76-78 have been deleted.

4. A mutant IL-2 molecule in which only amino acid residues 76-79 have been deleted.

5. A mutant IL-2 molecule in which only amino acid residue 75 has been deleted.

6. A mutant IL-2 molecule in which only amino acid residue 78 has been deleted.

7. A mutant IL-2 molecule in which only amino acid residues 75-77 have been deleted.

8. A mutant IL-2 molecule in which only amino acid residue 79 has been deleted.

9. A DNA sequence encoding the mutant IL-2 molecule of any of claims 1-8.

10. The DNA sequence of claim 9, contained in an expression vector.

11. A cell containing the expression vector of claim 10.

12. The DNA sequence of claim 9 wherein said DNA sequence is a synthetic sequence containing more prokaryotic preferred translation codons than naturally occurring IL-2 encoding DNA.

13. A method of producing mutant IL-2 comprising culturing the cell of claim 12 and recovering mutant IL-2 therefrom.

14. The mutant IL-2 molecule of any of claims 1-8, covalently linked to a portion of a toxin molecule which is large enough to exhibit cytotoxic activity and small enough to fail to exhibit generalized eukaryotic cell binding.

15. The molecule of claim 14 wherein said toxin molecule is diptheria toxin, and said portion of diptheria toxin is linked to said mutant IL-2 molecule by a peptide bond.

```
                        1                                   10
       IL-2 amino acid: Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr Gln Leu Gln
         cDNA sequence: gca cct act tca agt tct aca aag aaa aca cag cta caa
  codon modifications:                t   c       c           c       g   g
synthetic DNA sequence: GCA CCT ACT TCT AGC TCT ACC AAG AAA ACC CAG CTG CAG

                        20                                      30
Leu Glu His Leu Leu Leu Asp Leu Gln Met Ile Leu Asn Gly Ile Asn Asn Tyr Lys Asn
ctg gag cat tta ctg ctg gat tta cag alg att ttg aat gga att aat aat tac aag aat
          c c g               g           c c     c   t   c   c               c
CTC GAG CAC CTG CTG CTG GAT TTG CAG ATG ATC CTG AAC GGT ATC AAC AAT TAC AAG AAC
 XhoI

                        40                                      50
Pro Lys Leu Thr Arg Met Leu Thr Phe Lys Phe Tyr Met Pro Lys Lys Ala Thr Glu Leu
ccc aaa ctc acc agg atg ctc aca ttt aag ttt tac atg ccc aag aag gcc aca gaa ctg
  g       g     c t       g   c   c       c           g               c
CCG AAA CTG ACG CGT ATG CTG ACC TTC AAG TTC TAC ATG CCG AAG AAG GCC ACC GAA CTG
             MluI

                        60                                      70
Lys His Leu Gln Cys Leu Glu Glu Glu Leu Lys Pro Leu Glu Glu Val Leu Asn Leu Ala
aaa cat ctt cag tgt cta gaa gaa gaa ctc aaa cct ctg gag gaa gtg cta aat tta gct
      c   g           g               g       g               t   g   c c g
AAA CAC CTG GAG TGT CTA GAA GAA GAA CTG AAA CCG CTG GAG GAA GTT CTG AAC CTG GCT
                    XbaI
```

FIG. 1-1

```
                                  80                                                  90
Gln Ser Lys Asn Phe Hls Leu Arg Pro Arg Asp Leu Ile Ser Asn Ile Asn Val Ile Val
caa agc aaa aac ttt cac tta aga ccc agg gac tta atc agc aat atc aac gta ata gtt
  g tct           c     c g  ct   g c t     c g     tct   c
CAG TCT AAA AAC TTC CAC CTG CGG CCG CGT GAC CTG ATC TCT AAC ATC AAC GTA ATC GTT
                           NoII

                                 100                                                 110
Leu Glu Leu Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala Asp Glu Thr Ala Thr
ctg gaa cta aag gga tct gaa aca aca ttc atg tgt gaa tat gct gat gag aca gca acc
         g       t         c   c                     c                 c
CTG GAA CTG AAG GGC TCT GAA ACC ACC TTC ATG TGT GAA TAC GCT GAT GAG ACC GCA ACC
               BanII

                                 120                                                 130
Ile Val Glu Phe Leu Asn Arg Trp Ile Thr Phe Cys Gln Ser Ile Ile Ser Thr Leu Thr STOP
att gta gaa ttt ctg aac aga tgg att acc ttt tgt caa agc atc atc tca aca cta act tga
  c         c         c t       c       c       g tct             t   c   g   c
ATC GTA GAA TTC CTG AAC CGT TGG ATC ACC TTC TGT CAG TCT ATC ATC TCT ACC CTG ACC TGA
       EcoRI
```

FIG. 1-2

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/04258

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07K 13/00; C12P 21/02

U.S.CL: 530/351; 435/69.5, 69.52

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	530/351, 435/69.5, 69.52

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Computer data base search on CAS and dialog for:
IL-2 and delet? and mutat? and amino acids no. 74-79

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X Y	Science, vol. 234, issued 17 October 1986, Cohen et al "Structure-Activity Studies of Interleukin-2," pages 349-51, see page 351.	2-4, 7 / 1-8
X y	PCT, A, WO/85/00817 (Souza et al), 28 February 1985, see claims.	1-8 / 1-8
X	Science, vol. 238, issued 18 December 1987, Brandhuber et al, "Three Dimensional Structure of Interleukin-2," pages 1707-09, see entire document.	1-8
X	The Journal of Biological Chemistry, vol. 262, No. 12, issued 25 April 1987, Ju et al, "Structure-Function Analysis of Human Interleukin-2", pages 5723-31, see entire document.	1-6

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
6 November 1990	10 JAN 1991
International Searching Authority [1]	**Signature of Authorized Officer** [20]
ISA / US	Garnette D. Draper, Prim. Exm.

Form PCT/ISA/210 (second sheet) (May 1986)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
X	Gene, vol. 34, issued 1985, Wells et al "Cassette Mutagenesis: An efficient Method for Generation of Multiple Mutations at Defined Sites," pages 315-23, see entire document.	1-8
X	Nucleic Acids Research, vol. 10, No. 20, issued 1982, Zoller et al, "Oligonucleotide-directed Mutagenesis Using M13-derived Vectors: an efficient and General Procedure for the Production of Point Mutations in any Fragment of DNA," pages 6487-6500, see entire document.	1-8

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers .... because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows: Group I, claims 1-8 to IL-2 muteins, classified 530/351; Group II, claims 9-13, to DNA, vectors, cells and method of making IL-2 mutein, classified 435/69.52 and 172.3; Group III, claims 14-15 to IL-2-toxin conjugates, classified 530/402.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-8 TELEPHONE PRACTICE

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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C07K 13/00, 17/00, 3/26
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A1

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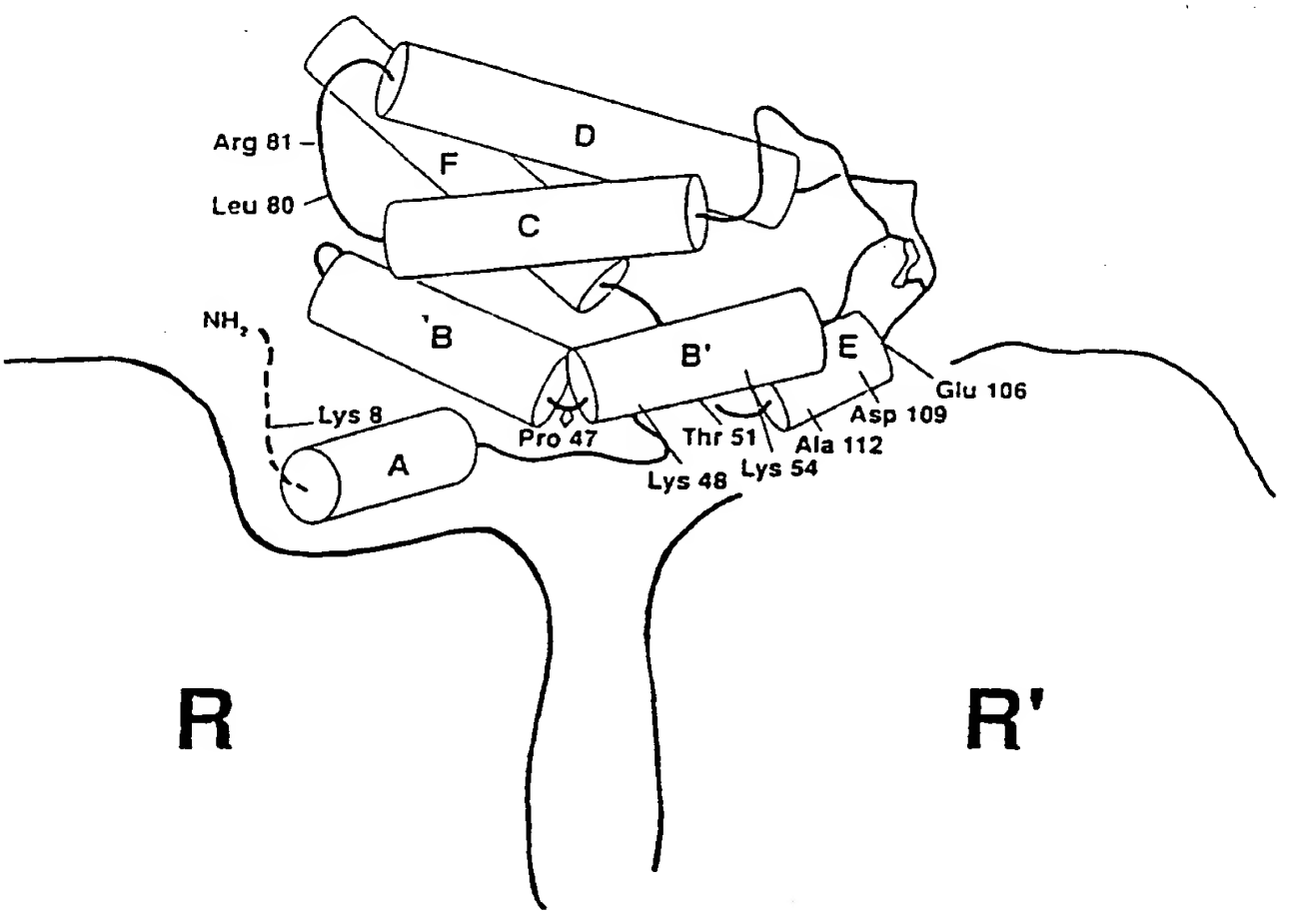
(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: INTERLEUKIN II ANALOGS



(57) Abstract

Interleukin II analogs and DNA sequences comprising structural genes coding for such analogs which differ from the naturally-occurring forms in terms of the identity and/or location of one or more amino acids are disclosed.

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INTERLEUKIN II ANALOGS

The present invention relates generally to the manipulation of genetic materials and, more particularly, to the manufacture of specific DNA sequences useful in recombinant procedures to secure expression of Interleukin II analogs.

Background of the Invention

Interleukin II ("IL-2"), a glycoprotein with a molecular weight of approximately 15,000, is a member of a group of proteins, called lymphokines, that control the body's immune response. IL-2 is produced by certain white blood cells, lectin- or antigen-activated T cells, and plays a central role in the body's immune system as a lymphocyte regulating molecule.

IL-2 has been reported to enhance thymocyte mitogenesis, to stimulate long-term in vitro growth of activated T-cell clones, to induce cytotoxic T-cell reactivity, to modulate immunological effects on activated B cells and lymphokine activated cells, to induce plaque-forming cell responses in cultures of nude mouse spleen cells, and to regulate production of gamma interferon. It also augments natural killer cell activity and mediates the recovery of the immune function of lymphocytes in selected immunodeficient states.

Additionally, in the laboratory, IL-2 is used to maintain cultures of functional monoclonal T-cells to study the molecular nature of T-cell differentiation, and to help elicit the mechanism of differentiated T-cell functions. Thus, IL-2 has application in both research and the treatment of neoplastic and immunodeficiency diseases.

IL-2 asserts its effect by binding to a specific high affinity receptor on the surface of target cells; consequently, the IL-2 molecule has become a focal point for studying receptor-effector interactions that modulate cell proliferation in the immune response.

The high affinity (K_D -10^{-11}M) receptor reponsible for mediating the effect of IL-2 on target cells consists of two distinct membrane-bound proteins of size 55 kD (p55 or Tac) and 75 kD (p75); each of these two proteins can act by itself as an apparent low affinity (K_D -10^{-8}M) receptor for IL-2, and both are required for IL-2 activity. This suggests that IL-2 must bind both p55 and p75 to form a trimeric complex for activity, and by inference, that IL-2 must have two separate receptor binding sites.

The limited amount of purified native IL-2 obtainable from peripheral blood lymphocytes and tumor cell lines was an impediment to studies of the biological role of this lymphokine until the advent of recombinant production of IL-2.

Taniguchi, T., et al., Nature, 302: 305-310 (1983) described the sequence analysis, cloning, and expression of a complementary DNA coding for human IL-2, cloned from a cDNA library prepared from partially purified IL-2 mRNA from the Jurkat leukemia cell line. IL-2 was proposed to comprise 133 amino acid residues and to have a calculated molecular weight of about 15,420. Taniguchi described the cloning procedures and the expression of the cDNA for IL-2 in cultured monkey COS cells. The publication states that expression of the IL-2 cDNA in E. coli had not yet been accomplished. See also European Patent Applications 118,617, published September 19, 1984; 118,977, published September 19, 1984; and 119,621, published September 26, 1984, and U.S. Patent 4,738,927.

Rosenberg, et al., Science, 223: 1412-1415 (1984) reported the isolation of another cDNA clone of the IL-2 gene from the Jurkat tumor cell line and from normal human peripheral blood lymphocytes. These researchers inserted the gene into E. coli, purified the polypeptide product and assayed it for biological activity. See also, Wang, et al., Science, 224, 1431-1433 (1984) referring to site-specific mutagenesis of a human IL-2 gene as well as European Patent Application 109,748, published May 30, 1984.

IL-2 modifications reported in the literature include: Ju et al., J. Biol. Chem. 262, 5723(1987); Liang et al., J. Biol. Chem. 261, 334(1986); and Miyaji et al., Agric. Biol. Chem., 51 1135(1987).

Considerable interest exists in the development of methods and materials for the production of large amounts of purified IL-2 analogs to replace IL-2-containing preparations currently employed in immunotherapy research.

It is an object of the subject invention to provide improved forms of IL-2.

It is a further object of the invention to provide IL-2 analogs having less toxicity than IL-2 preparations currently used.

It is a further object of the invention to provide IL-2 analogs which allow attachment of a ligand, without affecting biological activity.

It is a still further object of the invention to provide a method of purifying IL-2.

Other objects, features and characteristics of the present invention will become apparent upon consideration of the following description and the appended claims.

Summary of the Invention

The subject invention relates to IL-2 analogs having modified receptor domains, and analogs having stabilized IL-2 structure. The subject invention also relates to IL-2 analogs which have been modified to permit the attachment of a ligand. More particularly the subject invention relates to a polypeptide product of the expression in a host cell of a manufactured gene, the polypeptide having an amino acid sequence represented by formula [I] below wherein at least one of the 47th, 51st, 80th, 81st, 106th, 109th, 112th, 119th, 120th, 123rd, 127th, 129th, 131st, and 133rd original amino acid residues is replaced by a substitution amino acid residue, or wherein at least two of the 8th, 47th, 48th, 51st, 54th, 80th, 81st, 106th, 109th, 112th, 119th, 120th, 121st, 123rd, 127th, 129th, 130th, 131st, 132nd, and 133rd original amino acid residues are replaced by substitution amino acid residues, and/or an additional residue is attached at the carboxy terminus, and wherein X is selected from the group consisting of Cys, Ala, and Ser:

Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr
Gln Leu Gln Leu Glu His Leu Leu Leu Asp
Leu Gln Met Ile Leu Asn Gly Ile Asn Asn
Tyr Lys Asn Pro Lys Leu Thr Arg Met Leu
Thr Phe Lys Phe Tyr Met Pro Lys Lys Ala
Thr Glu Leu Lys His Leu Gln Cys Leu Glu [I]
Glu Glu Leu Lys Pro Leu Glu Glu Val Leu
Asn Leu Ala Gln Ser Lys Asn Phe His Leu
Arg Pro Arg Asp Leu Ile Ser Asn Ile Asn
Val Ile Val Leu Glu Leu Lys Gly Ser Glu
Thr Thr Phe Met Cys Glu Tyr Ala Asp Glu
Thr Ala Thr Ile Val Glu Phe Leu Asn Arg
Trp Ile Thr Phe X Gln Ser Ile Ile Ser
Thr Leu Thr

The invention also encompasses: IL-2 analogs wherein one, two, three or more original amino acids in any helix or in any helices of IL-2 (advantageously helix A and/or helix F) have been replaced by substitution amino acids which maintain or reduce the amphiphilicity of the helix or helices; analogs wherein one, two, three or more original amino acids in helix A, B, B' and/or E have been replaced by substitution amino acids, each having a different charge from the original amino acid it replaces; and analogs wherein one, two, three or more original amino acids in helix A, B, B', C, D, E and/or F have been replaced by substitution amino acids, each having a greater preference for alpha-helical structure then the original amino acid it replaces. The invention also relates to manufactured DNA sequences encoding such polypeptides. Further, the invention relates to monoclonal antibodies specifically binding such peptides, and to methods of purifying IL-2 and IL-2 analogs.

Brief Description of the Drawings

Figure 1(a) represents the alpha carbon backbone of IL-2. Figure 1(b) is a schematic stereo drawing of IL-2; helices are represented as cylinders.

Figure 2 is a schematic drawing showing a possible mode of interaction of IL-2 with its receptors.

Figure 3 shows the IL-2 structure and the positions of relevant amino acids.

Detailed Description of the Invention

Novel polypeptide analogs of Interleukin II ("IL-2") have been discovered. In a first embodiment of the invention, site specific modifications of the proposed receptor binding domains of naturally occurring IL-2 are made, and alterations which stabilize IL-2

helix structure and the overall IL-2 structure, are made. In a second embodiment, an amino acid such as an odd cysteine is incorporated into IL-2 at a location far removed from the proposed receptor binding domains but accessible to chemical reaction with other molecules.

Also provided by the present invention are manufactured genes capable of directing synthesis, in selected microbial hosts (e.g., bacteria, yeast and mammalian cells in culture), of the above noted IL-2 analogs. In preferred forms of manufactured genes, the base sequence includes one or more codons selected from among alternative codons specifying the same amino acid on the basis of preferential expression characteristics for the codon in a projected host microorganism, e.g., E. coli (see Alton et al., PCT application WO 83/04053).

Other preferred forms of manufactured genes include those wherein there is provided the nucleotide bases for a codon specifying an additional amino acid residue in the polypeptide coded for, which facilitates the direct expression in E. coli organisms (e.g., an initial Met residue). In still other preferred forms of manufactured genes, the base sequence of codons specifying the desired polypeptide is preceded by and/or followed by and/or includes one or more sequences of bases facilitating formation of expression vectors or generation of new structural genes for polypeptide analogs, i.e., sequences of bases providing for selected restriction endonuclease cleavage sites on one or both ends of the structural gene or at intermediate positions therein, and sequences providing a site for ribosome binding, e.g. CAA GGA GGT.

Also provided by the present invention are manufactured genes capable of directing the microbial expression of IL-2 analogs which differ from the naturally-occurring polypeptide in terms of the identity and/or location of one or more amino acid residues.

In the practice of the invention, manufactured DNA sequences are inserted into viral or circular plasmid DNA vectors to form hybrid vectors and the hybrid vectors are employed to transform microbial hosts such as bacteria (e.g., E. coli), yeast cells, or mammalian cells in culture. The transformed microorganisms are thereafter grown under appropriate nutrient conditions and express the polypeptide products of the invention.

Also comprehended by the invention are pharmaceutical compositions comprising effective amounts of polypeptide products of the invention together with suitable diluents, adjuvants and/or carriers useful in IL-2 therapy.

As employed herein, the term "manufactured" as applied to a DNA sequence or gene shall designate a product chemically synthesized by assembly of nucleotide bases, synthesized by site-directed mutagenesis, or derived from the biological replication of a product thus synthesized. As such, the term is exclusive of products "synthesized" by cDNA methods or genomic cloning methodologies which involve materials which are of biological origin.

As employed herein the term "substitution amino acid" means an amino acid which replaces the naturally occurring ("original") amino acid, and which is different from the original amino acid.

In another embodiment of the invention, antibodies are provided which specifically bind the polypeptides of the subject invention but which do not cross-react with naturally occurring IL-2. These antibodies can be tagged using methods known to those skilled in the art.

The following abbreviations shall be employed herein to designate amino acids: Alanine, Ala; Arginine, Arg; Asparagine, Asn; Aspartic acid, Asp;

Cysteine, Cys; Glutamine, Gln; Glutamic acid, Glu; Glycine, Gly; Histidine, His; Isoleucine, Ile; Leucine, Leu; Lysine, Lys; Methionine, Met; Phenylalanine, Phe; Proline, Pro; Serine, Ser; Threonine, Thr; Tryptophan, Trp; Tyrosine, Tyr; Valine, Val. The following abbreviations shall be employed for nucleotide bases: A for adenine; G for guanine; T for thymine; U for uracil; and C for cytosine.

While not wishing to be constrained to any particular theory of operation of the invention, the following detailed description is presented.

It has now been established that IL-2 is an alpha-helical protein (Fig. 1), Brandhuber et al., Science, 238, 1707 (1987) hereby incorporated by reference. It has a short helical segment near the amino terminus (residues 11 to 19; helix A in Fig. 1), followed by an extended loop; residues 33 to 56 form a helix interrupted, or "bent," near the middle by Pro 47 (hence the two segments are referenced as B and B'); following Cys 58 of the disulfide are helix C, residues 66 to 78, and D, residues 83 to 101; following Cys 105 is a short, apparently helical stretch E, residues 106 to 113, which leads into the carboxyl-terminal helix F, residues 117 to 133. There are no apparent segments of β-secondary structure in the molecule. The overall helical content of about 65 percent is in good agreement with estimates based on circular dichroism. The disulfide between Cys 58 and Cys 105 links two extending loops that connect the helices across the "top" (in the orientation of Fig. 1) of the molecule.

Helices B, C, D, and F form an antiparallel alpha helical bundle which differs significantly from the classical four-helix bundle represented by cytochrome c', cytochrome b_{562}, and myohemerythrin. The packing regions of the helices are shorter, involving only three to four turns of helix, while classical four-

helix bundles usually have at least five turns in each helix. Further, the packing angles all fall in the range of 25° to 30°, and hence are somewhat larger than the average of approximately 18° found in classical four-helix bundles.

Murine IL-2 is expected to have a similar structure to recombinant human IL-2 beginning with helix A and including the proline-induced bend in helix B+B'. This is significant since recombinant human IL-2 shows activity on both human and murine T cells, and recombinant murine IL-2 is reported to have a low but measurable activity on human T cells. The murine and human IL-2 sequences have 64 percent overall homology. The amino acid sequence of the mature murine protein is identical to the human sequence for the first seven residues, and then has one or more insertions, a total of 15 amino acids, relative to human, including a 12-residue poly (Gln) stretch, prior to Leu 14 of human IL-2; hence the amino terminal region of the murine protein may have significant structural differences from the human protein up to, and possibly including the first turn of, helix A. The only additional insertion in the murine sequence is between human IL-2 residues 80 and 81, in the loop connecting helices C and D.

The current data on IL-2 receptor binding suggest that the molecule "bridges" two receptor molecules, p55 and p75, with two independent binding sites, when bound to its high affinity receptor. Earlier work did not presage the presence of two receptor molecules; hence, modifications that affect IL-2 receptor binding do not discriminate between those involving a p55-(IL-2) interaction, a p75-(IL-2) interaction, or both.

Antibodies to peptides that cross-react with IL-2 have been used to map global regions in the IL-2 sequence likely to be important in receptor binding. In

particular, Kuo and Robb have presented evidence suggesting regions within the residue bounds 8 to 27 and 33 to 54 are directly involved in receptor binding, L. Kuo and R. J. Robb, J. Immunol. 137, 1538 (1986), while Altman and colleagues found that antibodies against peptides of residues 59 to 72, 91 to 105, and 119 to 133 did not inhibit IL-2 receptor binding, A. Altman, et al., Proc. Natl. Acad. Sci. U.S.A. 81, 2176 (1984).

Ju et al. supra, have demonstrated that deletion of residues 1 to 10 of human IL-2 (the amino terminus to the beginning of helix A) reduces induction of proliferation of murine CTLL-2 cells by only 30 to 50 percent, whereas deletion of residues 1 to 20 (the amino terminus including helix A) abolishes activity completely, Ju et al., supra. Deletion analysis of murine IL-2 shows a similar pattern of effects on proliferation activity of murine HT2 T cells, S. M. Zurawski et al., J. Immunol. 137, 3354 (1986). Deletion of murine residues 1 to 11 or 1 to 13 (prior to helix A, assuming murine IL-2 is structurally similar to human IL-2) reduces activity by at most 50 percent. Deletions of the murine poly (Gln) section, residues 15 to 26, coupled with various changes in sequence in the first 37 amino acids, has resulted in mutant protein with as much as one-third the specific activity of the native protein. However, deletion through murine residue 30 (corresponding to human residue 16, in the middle of helix A) reduces activity to about 0.4 percent that of the native protein, and deletion through residue 41 (corresponding to human residue 27) abolishes activity completely.

Most of the other reported deletions that abolish activity - many of which would delete a significant fraction of an internal helix in the structure or the peptide connecting them - are such that they may disrupt the overall tertiary structure of IL-2.

Data on site-specific amino acid substitutions suffer from lack of distinction between those mutations that affect activity by destabilizing the IL-2 structure and those that directly affect receptor binding. Except for alterations that destroy the disulfide of IL-2 or modify Trp 121 (whose side chain is internal in the structure), all of the mutations shown to lower activity of human IL-2 are in sequence regions 3 to 17 and 36 to 54, Ju et al. supra, which corroborates the receptor binding regions suggested by antibody competition studies. The "down" point mutations, when placed on the IL-2 model, do not identify a specific receptor binding surface.

It is believed that Helices B, C, D, and F form a structural scaffold, and that helices A, B' and part of B, and E form the receptor binding sites of IL-2 (Fig. 2). The involvement of helix E is suggested primarily by its spatial accessibility and its proximity to regions of the molecule probably involved in receptor interactions.

IL-2 binds, through a high affinity receptor, to T-cells but will also bind and activate other immune system cells through lower affinity receptors. It is believed that activation of these other cells contributes to the observed toxic side effects of IL-2.

Structural Variants of Human IL-2

Alterations of the receptor binding domains of IL-2 and alterations which stabilize the IL-2 structure, produce IL-2 analogs of altered specificity towards T-cells and results in an improved IL-2 molecule possessing altered activity and/or toxicity. Included in the subject invention are IL-2 analogs wherein amino acids, advantageously hydrophilic amino acids in helices in the receptor binding domain, are replaced by amino

acids having a different charge (e.g. replacing an amino acid having a positively charged side chain by an amino acid having a negatively charged side chain, or by an amino acid having an uncharged side chain, see below). Also encompassed by the subject invention are analogs wherein one or more amino acids which have a preference for α-helical structure (see Chou and Fasman, Annu. Rev. Biochem. 47, 251(1978).

have been substituted into one or more of the helices of IL-2, particularly helices A, B', B, E and F, in order to stabilize the structure of the helix and of the analog as a whole. For ease in understanding the present invention, the Chou and Fasman hierarchy is presented below:

Preference for Forming α Helix

Glu(-)	1.51	
Met	1.45	H_α = strong α former
Ala	1.42	
Leu	1.21	
Lys(+)	1.16	
Phe	1.13	
Gln	1.11	h_α = α former
Trp	1.08	
Ile	1.08	
Val	1.06	
Asp(-)	1.01	I_α = weak α former
His(+)	1.00	
Arg(+)	0.98	
Thr	0.83	i_α = α indifferent
Ser	0.77	
Cys	0.70	
Tyr	0.69	b_α = α breaker
Asn	0.67	
Pro	0.57	B_α = strong α breaker
Gly	0.57	

The helices of IL-2 are amphiphilic helices (see Kaiser et al., PNAS, 80, 1137-1143(1983) and Kaiser et al., Science, 223, 249-255(1984).

This amphiphilic helical structure is shared by several cytotoxic peptides such as mellitin, pardoxin, and maganins. More specifically, the F helix is very amphiphilic and some of the amino acids in the F helix do not have a strong preference for the α-helical structure, and it is believed that the interaction of the hydrophobic face of the F helix with the C and D helices provides the energy required to maintain the F helix sequence in its helical form. IL-2 analogs have been constructed containing altered helix sequences in which the amino acid replacements were selected to contain residues with a greater preference for a α-helical structure (e.g. Asn → Gln, Trp → Phe, Ser → Gln) and consequently the helices do not require strong amphiphilic interactions to maintain their helical structure, and thus the amphiphilicity of the helix can be maintained (for example an amino acid having a hydrophilic side chain being changed to a different amino acid having a hydrophilic side chain - the three substitutions noted above maintain amphiphilicity) or altered (for example by replacement of an amino acid having a hydrophilic side chain with one having a hydrophobic side chain), by amino acid substitution.

Analogs have been constructed to which other molecules can be covalently attached without damaging activity. These analogs are used to attach toxins, reporter groups, or antiviral or other therapeutic compounds which lead to the development of IL-2 conjugates of therapeutic importance as well as the production of specifically labeled IL-2 for the development of sensitive biological assays. The most convenient way to achieve these site specific conjugations is through the introduction of an odd

cysteine residue which will present a unique reactive sulphydryl group. Since the naturally occurring odd cysteine (Cys 125), is buried and most likely unreactive, the strategy focuses on the incorporation of a chemically accessible cysteine into (Ala^{125})IL-2.

The subject invention includes alterations where an additional amino acid is inserted between existing amino acids as an alternative to replacement of an existing amino acid.

I. Alterations in the Receptor Binding Domain of IL-2 and Alterations which Stabilized the IL-2 Structure

Hydrophilic amino acids in alpha helical regions (designated A, B', B and E in Figures 1, 2 and 3), implicated in high affinity receptor interactions, are among the candidate locations for amino acid changes. Substitution of other amino acids (particularly amino acids which alter the charge on the helix surface which interacts with the receptor) at these sites alter the spectrum of activities of the molecule by affecting receptor binding properties. Such changes also alter the ability of the molecule to interact with receptors on different cell types. Those cells are then more or less susceptable to IL-2 depending on the character of their surface IL-2 receptors. For example, cells bearing only the p75 component of the IL-2 receptor do not respond to an IL-2 species wherein the p75 binding domain has been altered such that the molecule can no longer bind to the receptor in a biologically meaningful way. The introduction of such selectivity to the IL-2 cell stimulation process allows for a greater therapeutic index for the material (e.g. by a reduction in undesirable side effects which results from stimulation of one cell type while at the same time retaining the ability of IL-2 to stimulate an appropriate effector cell

type which, in turn, limits disease). For example, certain alterations in helices in the receptor binding domain produce IL-2 analogs having a reduced capacity to induce induction of lymphokines such as INF-γ, IL-1 and TNF, but having equivalent biological activity, relative to (Ala^{125})IL-2 or natural IL-2.

For ease in understanding the present invention, the side chains of the following amino acids are generally considered to be nonpolar (hydrophobic):

Ile, Leu, Met, Phe, Pro, Trp, Tyr, and Val;

the side chains of the following amino acids are polar (hydrophilic) but uncharged:

Ala, Asn, Cys, Gln, Gly, Ser, and Thr;

the side chains of the following amino acids are hydrophilic and positively charged:

Arg, His, and Lys;

and the side chains of the following amino acids are hydrophilic and negatively charged:

Asp and Glu.

See also Hopp and Woods, PNAS 78 No. 6, 3824-3828(1981); Kyte and Doolittle, J. Molec. Biol., 157, 105-132(1982); and Parker et al., Biochemistry, 25 5425-5432(1986).

A. Alterations of the E Helix

The short E helix is involved in receptor binding. Therefore, the E helix is an excellent target for mutations that alter receptor binding. The side groups of three amino acids, Glu 106, Asp 109, and Ala 112, protrude out from the E helix for possible interaction with the receptor. Figure 3 shows the IL-2 structure, the proposed receptor binding domains, and positions of the relevant amino acids. Receptor - effector (e.g. IL-2) interactions are in part mediated by electrostatic interactions between charged groups on

the receptor with oppositely charged groups on the effector. The E helix has no positively charged amino acid side chains and three negatively charged side chains (Glu 106, Asp 109 and Glu 110).Alterations of the type of charge in the E helix of IL-2 thus alters binding efficiency. An Asp 109 →Lys 109 substitution alters receptor binding with a single mutation. Asp 109 protrudes from the center of the E helix and is a conserved amino acid. A Glu 106, Asp 109, Ala 112 →Lys 106, Lys 109, Lys 112 substitution radically changes the E helix from a negatively charged to positively charged surface. Changing either Glu 106 or Asp 109 (acidic residues) to Lys results in a net change in charge of +2 of the E helix. Changing a neutral residue (Ala 112) to Lys gives a change of +1. Changing all three residues to Lys results in a change in charge of +5. These changes can be made individually or together. Further, substitutions can be made with amino acids which have a greater preference for the α-helical structure.

B. Alterations of the B and B' Helices

Three amino acids, Lys 48, Thr 51, and Lys 54 protrude out from the B' helix for possible interaction with receptor. The B' helix has one negatively charged amino acid side chain (Glu 52), and four positively charged side chains (Lys 48, Lys 49, Lys 54, and His 55). A Lys 48, Thr 51, Lys 54 →Glu 48, Asp 51, Glu 54 substitution radically changes the B' helix from a positively charged to negatively charged surface. Similar changes in charge can be made in the B helix. As in the case with the E helix alterations, these changes can be done individually or together. As with the other helices of IL-2, substitutions can be made with amino acids which have a greater preference for the α-helical structure.

C. Pro 47 -Gly 47

The breakage of the B helix into the B and B' helices is a unique structural feature of IL-2. It is believed that Pro 47 rigidly holds B and B' helices at the optimum angle to allow the B' residues to interact properly with the receptor. A Pro 47 -Gly 47 substitution gives more flexibility to the hinge joint separating the B and B' helices which changes the positioning of the important B' residues and ultimately alters the IL-2-receptor interaction. Substitutions by other amino acids at position 47 are also encompassed by the subject invention.

D. Alterations of the A Helix

The A helix is involved in receptor binding. The A helix has one negatively charged amino acid side chain (Glu 115), and one positively charged side chain (His 16). As with the the other helices of IL-2, amino acids which have a preference for the α-helical structure can be substituted to strengthen the structure of the A helix. Alteration of charge in the A helix as in the E, B, and B' helices, is also encompassed by the subject invention. Lastly, amino acid changes which maintain or reduce the amphiphilicity of the A helix are also included in the subject invention.

E. Alteration of the F Helix

The F helix is an amphiphilic helix. Some of the amino acids in the F helix do not have a strong preference for the α-helical structure and it is believed that the interaction of the hydrophobic face of the F helix with the C and D helices provides the energy required to maintain the F helix in its helical form.

IL-2 analogs have been constructed containing altered F helix sequences in which the amino acid replacements were selected to contain residues with a higher preference for an α-helical structure (e.g. Asn → Gln, Trp → Phe, Ser → Gln) and consequently the F helix does not require strong amphiphilic interactions to maintain its helical structure, and thus the amphiphilicity of the helix can be maintained or reduced.

II. Alterations in IL-2 Which Allow Attachment of a Ligand

The sites of substitutions which allow attachment of a ligand include the carboxyl terminus as well as portions of the molecule with surface exposure chosen such as to minimally perturb the structure of active IL-2. For example, addition, insertion or substitution of a cysteine residue provides a sulphydryl group which can be chemically conjugated to:

a) radiolabeled moieties (for assay, imaging). Conjugation of reporter groups to IL-2 allow for the rapid and sensitive detection of the resulting active IL-2 analogs. These conjugations are used as the key component in the development of sensitive IL-2 biological assays;

b) enzymatic moieties (for assay, directed therapeutic delivery);

c) toxins (for selective cell killing, in vitro or in vivo). Conjugation of IL-2 to cytotoxic agents should direct the toxins to cells which present IL-2 receptors. These conjugations are useful in treating certain leukemias, transplant rejections, autoimmune or altered immune states, or other cell populations of pathological significance;

d) drugs (directed therapeutic delivery, e.g. AZT for AIDS). Cells that are susceptible to HIV infection are, for the most part, the cells that carry

IL-2 receptors. Conjugation of IL-2 to retrovirus inhibitors, such as AZT (a reverse transcriptase inhibitor) direct the inhibitor to the infected cells and provide a mechanism for the internalization of the inhibitor through the IL-2 receptor. These conjugations are useful in treating AIDS and other related diseases;

e) antibodies or mitogens (selective cell targeting, e.g. helper T cells using OKT 4 or equivalent, and/or selective cell activation to an IL-2 responsive state); or the like.

Examination of the x-ray structure of IL-2 reveals that it is possible to conjugate other molecules to IL-2 either at the carboxy terminus or in the region spanning amino acids 79 to 82, without interfering with the receptor binding domain of the IL-2 molecule (see Figure 3). A free cysteine residue has been incorporated into (Ala^{125})IL-2 (an IL-2 analog containing one disulfide bond but no free cysteines) at the carboxy terminus and/or the 79 to 82 region of IL-2. Incorporation of the free cysteine residue(s) at these positions, accomplished by modifying the recombinant IL-2 gene, allows for the specific chemical conjugation of other molecules to IL-2, by reaction with the free sulphydryl group(s), in a manner that does not affect the binding of IL-2 to its cell surface receptors.

A. Alterations in the Carboxy Terminus Region

The carboxy terminus region of IL-2 is not involved in receptor binding and is a good location for the incorporation of an odd cysteine. Leu 132 was chosen because it is close to the C-terminus (next to last amino acid) and it is an unconserved residue when comparing human, bovine, and murine IL-2 sequences. An analog in which cysteine is simply added to the carboxy end of IL-2 ((Cys^{134})IL-2) also accomplishes the goal of incorporating an odd cysteine at the C-terminus.

B. Alterations in the Region Between C and D Helices

The alignment of human, bovine, and murine IL-2 sequences show that, relative to the human sequence, the bovine and murine sequences contain an insertion between amino acids 80 and 81. The amino acids are part of a four amino acid loop (amino acids 79 to 82) that connects the C and D helices of IL-2. This observation coupled with the fact that this region of the molecule is far removed from the proposed receptor binding domains and other Cys residues in the molecule, makes this an ideal location for an insertion of an odd cysteine residue. Substitutions such as Leu 80 →Cys 80 are also encompassed by the invention.

C. Alterations in the Amino-Terminal Region

A Lys 8 →Cys 8 substitution at a nonconserved residue provides a reactive group near the amino terminus.

IV. Active or Competitive IL-2 Fragments

Some structural component or combination of components of IL-2 have retained or lost biological properties of intact IL-2 together with the ability to bind IL-2 receptor(s). Such a peptide is useful in place of intact IL-2 or as an antagonist of its action(s). Peptides for this application include at least one of the A, B, B' and E helices, for example, the invention includes A and E helical regions which preserve their own internal symmetry as species isolated and apart from the conformational constraints of the intact parent molecule. Such isolated structures bind to components of the IL-2 receptor and either do or do not have IL-2 biological activity. Such structures retain activity on

only a subset of IL-2 responsive cells allowing for greater precision in manipulating the IL-2 response. Such isolated structures have lost biological activity but function as competitive inhibitors of IL-2 binding and are useful in antagonizing physiological states involving a stimulant effect of IL-2. Such structures function to up or down regulate IL-2 receptors and, thereby influence cellular receptivity to IL-2.

V. Additional Alterations

Additional analogs which are encompassed by the present invention include the analogs of IL-2 noted above further characterized by the presence of one or more of the following alterations in the amino acid sequence of naturally-occurring IL-2.

(a) deletion and/or replacement of amino acid residues providing sites of intramolecular folding;

(b) deletion of terminal amino acid residues;

(c) addition of amino acid residues to terminal amino acid residues;

(d) deletion and/or replacement of amino acid residues providing sites of hydrolytic instability under highly acidic conditions;

(e) replacement of amino acid residues with glutamine residues;

(f) replacement of amino acid residues with phenylalanine residues;

(g) deletion and/or replacement of tryptophan residues;

(h) deletion and/or replacement of asparagine residues;

(i) deletion and/or replacement of cysteine residues;

(j) replacement of amino acid residues with serine residues; and

(k) replacement of amino acid residues with alanine residues.

IL-2 Purification and Removal of Pyrogens

IL-2 and IL-2 analogs are very hydrophobic proteins and as such have a propensity to bind pyrogens which are also hydrophobic. These peptides can be formulated in a stable, monomeric form at acidic pH values. Under these conditions, pyrogens tend to form higher molecular weight aggregates, even though the monomeric molecular weight of pyrogens is comparable to that of IL-2. Thus, manufacturing procedures which fractionate proteins on the basis of size are used to separate monomeric IL-2 from aggregated forms of pyrogens. A suitable procedure for carrying out this step is by ultrafiltration through YM-30 (Amicon) membranes. Repeated dilution and ultrafiltration can be used to enhance the yield of IL-2. Glucose, mannitol, or another bulking agent, can be added as a toxicity modifier and the desired concentration of the IL-2 can be obtained by concentration by ultrafiltration or by dilution with an appropriate buffer. Pyrogens can also be separated from monomeric IL-2 by size exclusion chromatography, e.g. using Sephadex G-75. Detergents (e.g. laurate, sarcosine, sodium dodecyl sulfate) render this method ineffective since, in the presence of detergents, pyrogens are reduced to lower molecular weight forms and have apparent molecular weights comparable to IL-2. Procedures which might be expected to remove pyrogens from IL-2 solutions, such as ion exchange chromatography and hydrophobic chromatography proved to be ineffective under the conditions examined. The method of the subject invention is easy to scale up and is very cost effective.

The following examples illustrate practice of the invention in the manufacture of the DNA sequences coding for microbial expression of IL-2 and polypeptide analogs thereof. Also illustrated is the construction of expression vectors for microbial expression of desired polypeptides.

EXAMPLE 1
Construction of Oligonucleotide Sequences

This example is directed to the procedure employed in the synthesis of oligonucleotide sequences employed to manufacture the IL-2 analog genes according to the invention.

Oligonucleotide sequences were synthesized using a four-step procedure with several intermediate washes. Syntheses were performed on Applied Biosystems (ABI) Model 380 automated synthesizers using ABI supplied reagents. Polymer bound dimethoxyltrityl protected nucleoside in support columns was first stripped of its 5'-protecting group (dimethoxyltrityl) using 3% trichloroacetic acid in dichloromethane for one minute. The polymer was then washed with acetonitrile. The washed polymer was then rinsed with dry acetonitrile, placed under argon and then treated in the condensation step using tetrazole in acetonitrile with the protected nucleoside phosphoramidite in acetonitrile. This reaction was allowed to proceed for 2.0 minutes. The reactants were then removed by filtration. This was followed by capping the unreacted 5 '-hydroxyl groups using a solution prepared by mixing one part of a mixture containing acetic anhydride, 2,6-lutidine and tetrahydrofuran (1:1:8), and one part 6.5% dimethylaminopyridine in tetrahydrofuran. After one minute the capping solution was removed and the polymer was treated for 1.5 minutes with an oxidizing solution (0.1 M I_2 in H_2O/2,6-lutidine/THF, 1:10:40).

This was followed by an acetonitrile rinse. The cycle began again with a trichloroacetic acid/methylene chloride deprotection and was repeated until the desired oligonucleotide sequence was obtained.

The final oligonucleotide chain was treated with fresh concentrated ammonia at room temperature for 2.0 hours. After decanting the solution from the polymer, the concentrated ammonia solution was heated at 60°C for 16 hours in a sealed tube.

Each oligonucleotide solution was extracted with 1-butanol and ethyl ether and the concentration of each solution was determined with a spectrophotometer (260nm). 5.0 OD units of each oligonucleotide were dried down for preparative electrophoresis and loaded into a 15% polyacrylamide, 7 molar urea gel. After electrophoresis, the product band was visualized by UV shadowing, cut from the gel, extracted and then desalted on a G-50 Sephadex column to yield the purified oligonucleotide.

EXAMPLE 2

Construction of IL-2 Analogs by Oligonucleotide Site-Directed Mutagenesis

This example relates to the use of recombinant methods to generate analogs of IL-2. Site directed mutagenesis procedures according to Souza, et al., published PCT Application No. WO 85/00817, published February 28, 1985,
were carried out on the DNA sequence shown in Table 1 (which has E. coli preference codons), using the oligonucleotides shown in Table 2.

TABLE 1

```
                                        -1   1
                                        MET  Ala  Pro  Thr  Ser  Ser  Ser  Thr  Lys
CTAGAAAAAC[CATGAGGGT]AATAAATA ATG  GCT  CCT  ACG  AGC  TCT  TCT  ACT  AAG

     10                      A                    20
Lys  Thr [Gln Leu  Gln  Leu  Glu  His  Leu  Leu  Leu] Asp  Leu  Gln  MET  Ile  Leu  Asn
AAA  ACC  CAG  CTG  CAA  CTG  GAA  CAT  CTG  CTT  CTT  GAC  CTG  CAA  ATG  ATC  CTG  AAC

               30                                B         40
Gly  Ile  Asn  Asn  Tyr  Lys [Asn  Pro  Lys  Leu  Thr  Arg  MET  Leu  Thr  Phe  Lys  Phe
GGT  ATC  AAC  AAC  TAC  AAA  AAC  CCG  AAG  CTT  ACC  CGT  ATG  CTG  ACT  TTC  AAA  TTC

                         50        (B')                                60
Tyr  MET] Pro [Lys  Lys  Ala  Thr  Glu  Leu  Lys  His  Leu] Gln  Cys  Leu  Glu  Glu  Glu
TAC  ATG  CCG  AAG  AAA  GCA  ACC  GAA  CTG  AAA  CAC  CTG  CAG  TGT  CTG  GAA  GAA  GAA

                              70        C                                     80
Leu  Lys  Pro [Leu  Glu  Glu  Val  Leu  Asn  Leu  Ala  Gln  Ser  Lys  Asn  Phe] His  Leu
CTG  AAA  CCT  CTG  GAG  GAA  GTT  TTA  AAC  CTG  GCT  CAA  TCC  AAG  AAC  TTT  CAT  CTG

                                        90   D
Arg  Pro [Arg  Asp  Leu  Ile  Ser  Asn  Ile  Asn  Val  Ile  Val  Leu  Glu  Leu  Lys  Gly
CGT  CCA  CGT  GAT  CTG  ATC  AGC  AAC  ATT  AAC  GTT  ATC  GTA  CTG  GAA  CTT  AAA  GGC

     100                                          110  E
Ser  Glu  Thr] Thr  Phe  MET  Cys [Glu  Tyr  Ala  Asp  Glu  Thr  Ala  Thr] Ile  Val  Glu
TCT  GAA  ACT  ACC  TTC  ATG  TGC  GAA  TAT  GCA  GAC  GAG  ACC  GCT  ACC  ATC  GTG  GAA

                                        125
               120                      Ala/GCT   F         130            133
[Phe Leu  Asn  Arg  Trp  Ile  Thr  Phe  Cys  Gln  Ser  Ile  Ile  Ser  Thr  Leu  Thr] STOP
TTT  CTG  AAT  CGT  TGG  ATC  ACT  TTC  TGT  CAG  TCC  ATC  ATC  AGC  ACT  CTG  ACC  TAA

STOP
TAG GATCCTAATAGTCGAC
```

TABLE 2

IL-2 ANALOGS	SEQUENCES (5' → 3')	Length
Leu^{80} →Cys^{80}	ACG TGG ACG GCA ATG AAA GTT CTT	24
Lys^{8} →Cys^{8}	GCT CTT CTA CTT GTA AAA CCC AGC	24
Pro^{47} →Gly^{47}	AAA TTC TAC ATG GGG AAG AAA GCA AC	26
Glu^{106} →Lys^{106}	GTC TGC ATA TTT GCA CAT GAA GG	23
Asp^{109} →Lys^{109}	AGC GGT CTC TTT TGC ATA TTC GC	23
Ala^{112} →Lys^{112}	CCA CGA TGG TTT TGG TCT CGT CT	23
Lys^{48} →Glu^{48}	CTA CAT GCC GGA GAA AGC AAC C	22
Thr^{51} →Asp^{51}	CGA AGA AAG CAG ACG AAC TGA AAC	24
Lys^{54} →Glu^{54}	ACC GAA CTG GAA CAC CTG CAG	21
→Cys^{134}	GGA TCC TAT TAG CAG GTC AGA GTG	24

Oligonucleotide site-directed mutagenesis was performed by cloning the IL-2 region from Xbal to BamHl, from expression vector pCFM 536 IL-2 into both M13mp10 and M13mp11, and the single-stranded phage DNA was isolated as for DNA sequencing. Although pCFM536 (see U.S. Patent 4,710,473 hereby incorporated by reference) was used, any suitable expression vector could have been used. This DNA was mixed with the synthetic deoxynucleotides of Table 2. The DNA in these mixtures was allowed to anneal by heating to 65°C and then slowly cooling to room temperature. The oligomers contained the appropriate base changes from the natural recombinant IL-2 sequence in the middle of their sequences. To the annealed DNAs were added ATP, dATP, dCTP, dGTP, TTP, T4 DNA ligase, and the Klenow fragment of E. coli DNA polymerase I. This reaction allowed the single-stranded primed phage DNAs to convert into covalently closed, double-stranded, circular DNAs. This DNA was transfected directly into E. coli strain JM103 without first purifying the in vitro synthesized double stranded DNA on alkaline sucrose gradients. Many of the plaques from the transfection contained phage DNA with the original recombinant IL-2 sequence, but some contained the IL-2 sequence with the desired base changes. These plaques were identified by lifting plaques onto nitrocellulose filters, and then hybridizing the filters with the synthetic deoxynucleotide end-labeled with ATP (γ-^{32}P). After hybridizing, the filters were washed at a temperature 0-3°C below the melting temperature of the synthetic deoxynucleotide and its complementary DNA sequence. These wash conditions selectively left strong autoradiography signals corresponding to plaques with phage containing the mutated sequence. Positive clones for each analog were confirmed by DNA sequencing, and these were cloned back into pCFM 536 from Xbal to BamHl.

Cultures of recombinant IL-2 analogs were grown in media containing 10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter at 30°C with shaking until they reached an A_{600} of 0.5 at which point they were rapidly heated to 42°C. The flasks were allowed to continue shaking at 42°C for three hours. Cells were harvested by centrifugation at 10,000 x G for 20 minutes at 4°C. Cell pellets were resuspended at 0.4 g wet weight/ml with 1 mM dithiothreitol (DTT) and were passed twice through a French Pressure Cell at 10,000 psi for 15 minutes at 4°C, and the broken cell supernatants were discarded. The pellets were resuspended in 50 mM Tris, 5 mM EDTA, 5 mM DTT, 0.5 M NaCl, 1% sodium deoxycholate (DOC), pH 9.0 at 0.25 g wet weight original pellet/ml and were allowed to mix for 30 minutes at room temperature. These mixtures were centrifuged at 10,000 x G for 15 minutes at 14°C and the supernatants were discarded. The pellets were resuspended in H_2O at 0.15 g wet weight original pellet/ml and centrifuged at 10,000 x G for 15 minutes at 4°C. The supernatants were discarded and the pellets were solubilized at room temperature in 4% sodium laurate, 50 mM Tris, 5% ethanol, 50mM DTT, pH 8.7 at approximately 20-30 mg protein/ml. The solubilized protein was chromatographed on Sephadex G-75 in 2% sodium laurate, 50 mM Tris, 5% ethanol, pH 8.7. Fractions were analyzed by SDS-PAGE and IL-2 containing fractions of greater than 95% purity were pooled.

Under certain circumstances it was desirable to have the IL-2 analogs essentially free of pyrogenic substances and endotoxins. This was accomplished by further purification of the molecule. The protein was oxidized in the presence of Cu^{2+}, concentrated and chromatographed on Sephadex G-75 equilibrated with 1% laurate/25 mM Tris/ 5% ethanol, pH 8.7. Those fractions containing monomeric forms of IL-2 were pooled and the

protein was precipitated by addition of an equal volume of ethanol. The pellet was collected by centrifugation, washed with 50% ethanol, and then solubilized in 50% acetic acid. The solution was diluted 50 fold to allow for refolding of the IL-2 analog, concentrated, then diafiltered against a sodium acetate buffer such that the final concentration and pH were 10 mM sodium acetate, pH 4.

EXAMPLE 3
Activity of the IL-2 Analogs

This example relates to the activity of analogs generated in Example 2.

A number of IL-2 analogs were generated that differ from the native sequence of IL-2 by two amino acids. All analogs tested have Ala at position 125. (Ala^{125})IL-2 (which differs from the native sequence of IL-2 by one amino acid) was used as a positive control for these experiments. (Asp^{51})IL-2 and (Glu^{48})IL-2 are molecules with amino acid changes in the putative receptor-binding B' domain of IL-2. Specifically, in (Asp^{51})IL-2 the neutral threonine at position 51 was replaced by a negatively charged aspartic acid, and in (Glu^{48})IL-2 the positively charged lysine was replaced by the negatively charged glutamic acid. In (Gly^{47})IL-2, the proline between the B and B' domains was replaced with a glycine. This change was predicted to have significant structural consequences for the molecule that would result in a substantial loss of biological activity. These analogs were tested in several <u>in vitro</u> assays for IL-2 activity - the incorporation of ^{3}H-thymidine into the murine T cell line CTLL-1 or into human peripheral blood leukocytes (hPBL), the generation of lymphokine activated killer cells (LAK cells) from hPBL cultures and for the ability

to induce IFN-gamma, IL-1 and TNF production in hPBL cultures. These experiments were executed with partially purified material. It is noted that in separate CTLL-1 assays using highly purified materials, the specific activity of (Ala^{125})IL-2 was found to be 7.8×10^6 U/mg, that for (Glu^{48})IL-2 was found to be 7.9×10^6 U/mg, and that for (Asp^{51}) IL-2 was found to be 6.6×10^6 U/mg. The data from several experiments is shown in Table 3 below.

TABLE 3
ASSAY

Exp.	Analog	CTLL-1 (Units/mg)	^{3}Hdtr into hPBL (cpm)	LAK cells (lytic unit/culture)	IFN-gamma (Units/ml)	IL-1beta (pg/ml)	TNF (pg/ml)
1	Ala(125)	$1.2x10^6$	25,987	-	440	-	-
	Asp(51)	$2.0x10^6$	37,546	-	160	-	-
	Glu(48)	$1.4x10^6$	25,983	-	100	-	-
	Gly(47)	$3x10^4$	5,788	-	25	-	-
2	Ala(125)	-	-	$42x10^3$	-	-	-
	Asp(51)	-	-	$47x10^3$	-	-	-
	Glu(48)	-	-	$49x10^3$	-	-	-
	Gly(47)	-	-	$7.4x10^3$	-	-	-
3	Ala(125)	$1.2x10^5$	-	$97x10^3$	94	527	4,640
	Asp(51)	$1.2x10^5$	-	$128x10^3$	55	610	5,600
	Glu(48)	$1.4x10^5$	-	$81x10^3$	48	290	2,080
	Gly(47)	$7.4x10^3$	-	$19x10^3$	16	130	720
4	Ala(125)	-	-	$266x10^3$	11,400	-	-
	Asp(51)	-	-	$266x10^3$	10,000	-	-
	Glu(48)	-	-	$375x10^3$	6,650	-	-
	Gly(47)	-	-	$35x10^3$	220	-	-

Taken together, these results indicate that (Asp^{51})IL-2 and (Glu^{48})IL-2 were equivalent to (Ala^{125})IL-2 as T cell mitogens and as inducers of LAK cells. However, (Glu^{48})IL-2 consistently induced less IFN-gamma production from hPBL cultures than did an equal concentration of (Ala^{125})IL-2. In two of three experiments, (Asp^{51})IL-2 was less effective than (Ala^{125})IL-2 in IFN-gamma induction. In one experiment, the production of IL-1 and TNF by the analogs was measured. The induction of these lymphokines by (Asp^{51})IL-2 was equal to that of (Ala^{125})IL-2 while induction by (Glu^{48})IL-2 was approximately 50% as effective. Depending on the assay, (Gly^{47})IL-2 had only 2% to 20% of the biological activity of (Ala^{125})IL-2 as predicted.

EXAMPLE 4

Alterations in the F-Helix to Include Residues with High Preference for Alpha-Helical Structure

The analogs containing altered F-helix sequence were constructed by a two step procedure. The first step involved the introduction of an Eco R1 restriction site at a region in the gene corresponding to the Glu^{116} Phe^{117} sequence. This was accomplished by site directed mutagenesis using the primer shown below which codes for the desired change in DNA sequence while leaving the encoded amino acid sequence intact.

Mutagenesis Primer for the Introduction of Eco R1 Site

	Glu^{116}	Phe^{117}	
5' - CGTG	GAA	TTC	CTGAATCGTT - 3'
		Eco R1	

The presence of the Eco R1 site allowed for the excision of the portion of the IL-2 gene coding for the F-helix by digestion with Eco R1 and Bam H1. The second step involved the replacement of the excised portion of the gene with the synthetic DNA sequence coding for the altered F-helix amino acid sequences. Using this method, the following IL-2 analogs C4 and C5 were constructed:

Natural Sequence

```
116                 120                 125                 130
GAA TTT CTG AAT CGT TGG ATC ACT TTC TGT CAG TCC ATC ATC AGC ACT CTG ACC
Glu Phe Leu Asn Arg Trp Ile Thr Phe Cys Gln Ser Ile Ile Ser Thr Leu Thr
```

Analog C4 ($Gln^{119}Lys^{120}Ala^{123}Ala^{127}Leu^{129}Ala^{131}$)IL-2

```
                                    125
GAA TTC CTG CAG AAA TGG ATC GCT TTC GCA CAG GCT ATC CTG AGC GCA CTG ACC
Glu Phe Leu Gln Lys Trp Ile Ala Phe Ala Gln Ala Ile Leu Ser Ala Leu Thr
```

Analog C5 ($Gln^{119}Lys^{120}Phe^{121}Ala^{123}Gln^{127}Leu^{129}Gln^{130}Ala^{131}Ala^{133}$) IL-2

```
                                    125
GAA TTC CTG CAG AAA TTC ATC GCT TTC GCA CAG CAG ATC CTG CAG GCA CTG GCT
Glu Phe Leu Gln Lys Phe Ile Ala Phe Ala Gln Gln Ile Leu Gln Ala Leu Ala
```

Natural Sequence Preferred Structure (A=α helix, B=β-sheet, T=turn)

```
Glu Phe Leu Asn Arg Trp Ile Thr Phe Cys Gln Ser Ile Ile Ser Thr Leu Thr
 T                   T   T   T   B   B   B   B   B   B   B   B   B   B
```

Analog C4 Structure

```
                                    125
Glu Phe Leu Gln Lys Trp Ile Ala Phe Ala Gln Ala Ile Leu Ser Ala Leu Thr
 A   A   A   A   A   A   A   A   A   A   A   A   A   A   A   A   A   A
```

Analog C5 Structure

```
                                    125
Glu Phe Leu Gln Lys Phe Ile Ala Phe Ala Gln Gln Ile Leu Gln Ala Leu Ala
 A   A   A   A   A   A   A   A   A   A   A   A   A   A   A   A   A   A
```

* * *

While the present invention has been described in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations which come within the scope of the invention as claimed.

WHAT IS CLAIMED IS:

1. A polypeptide product of the expression in a host cell of a manufactured gene, said polypeptide having an amino acid sequence represented by formula [I] below wherein at least one of the 47th, 51st, 80th, 81st, 106th, 109th, 112th, 119th, 120th, 123rd, 127th, 129th, 131st and 133rd original amino acid residue is replaced by a substitution amino acid residue, or wherein at least two of the 8th, 47th, 48th, 51st, 54th, 80th, 81st, 106th, 109th, 112th, 119th, 120th, 121st, 123rd, 127th, 129th, 130th, 131st, 132nd and 133rd original amino acid residues are replaced by substitution amino acid residues, and wherein X is selected from the group consisting of Cys, Ala, and Ser:

Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr
Gln Leu Gln Leu Glu His Leu Leu Leu Asp
Leu Gln Met Ile Leu Asn Gly Ile Asn Asn
Tyr Lys Asn Pro Lys Leu Thr Arg Met Leu
Thr Phe Lys Phe Tyr Met Pro Lys Lys Ala
Thr Glu Leu Lys His Leu Gln Cys Leu Glu [I]
Glu Glu Leu Lys Pro Leu Glu Glu Val Leu
Asn Leu Ala Gln Ser Lys Asn Phe His Leu
Arg Pro Arg Asp Leu Ile Ser Asn Ile Asn
Val Ile Val Leu Glu Leu Lys Gly Ser Glu
Thr Thr Phe Met Cys Glu Tyr Ala Asp Glu
Thr Ala Thr Ile Val Glu Phe Leu Asn Arg
Trp Ile Thr Phe X Gln Ser Ile Ile Ser
Thr Leu Thr.

2. A polypeptide according to Claim 1 characterized by the presence of one or more of the following substitutions in amino acid sequence:

$Lys^{8}\rightarrow Cys^{8}$;
$Pro^{47}\rightarrow Gly^{47}$;
$Thr^{51}\rightarrow Asp^{51}$;
$Leu^{80}\rightarrow Cys^{80}$;
$Arg^{81}\rightarrow Cys^{81}$;
$Glu^{106}\rightarrow Lys^{106}$;
$Asp^{109}\rightarrow Lys^{109}$;
$Ala^{112}\rightarrow Lys^{112}$;
$Asn^{119}\rightarrow Gln^{119}$;
$Arg^{120}\rightarrow Lys^{120}$;
$Thr^{123}\rightarrow Ala^{123}$;
$Ser^{127}\rightarrow Ala^{127}$ or Gln^{127};
$Ile^{129}\rightarrow Leu^{129}$;
$Ser^{130}\rightarrow Gln^{130}$;
$Thr^{131}\rightarrow Ala^{131}$;
$Leu^{132}\rightarrow Cys^{132}$;
$Thr^{133}\rightarrow Ala^{133}$;
addition of Cys^{134};

and optionally one or more of the following substitutions:

$Lys^{48}\rightarrow Gly^{48}$
$Lys^{54}\rightarrow Glu^{54}$
$Trp^{121}\rightarrow Phe^{121}$.

3. A polypeptide according to Claim 2 selected from the group consisting of:

(Cys^{8}) IL-2;
(Gly^{47}) IL-2;
(Asp^{51}) IL-2;
(Cys^{80}) IL-2;
(Cys^{81}) IL-2;
(Lys^{106}) IL-2;
(Lys^{109}) IL-2;
(Lys^{112}) IL-2;
(Cys^{132}) IL-2;
(Cys^{134}) IL-2;

4. A polypeptide according to Claim 1 wherein said polypeptide also has one or more of the following alterations in the amino acid sequence:

(a) deletion and/or replacement of amino acid residues providing sites of intramolecular folding;

(b) deletion of terminal amino acid residues;

(c) addition of amino acid residues to terminal amino acid residues;

(d) deletion and/or replacement of amino acid residues providing sites of hydrolytic instability under highly acidic conditions;

(e) replacement of amino acid residues with glutamine residues;

(f) replacement of amino acid residues with phenylalanine residues;

(g) deletion and/or replacement of tryptophan residues;

(h) deletion and/or replacement of asparagine residues;

(i) deletion and/or replacement of cysteine residues;

(j) replacement of amino acid residues with serine residues; and

(k) replacement of amino acid residues with alanine residues.

5. A polypeptide product according to Claim 1 in which at least one of: the 8th, 80th and 132nd amino acid residues is replaced by a substitution amino acid residue which permits attachment of a ligand, and/or a 134th amino acid residue which permits attachment of a ligand, is added.

6. A polypeptide as in Claim 5 wherein said substitution amino acid residue and said 134th amino acid residue are Cys.

7. A polypeptide as in Claim 5 coupled via said 8th, 80th, 81st, 132nd or 134th amino acid residue, to a label.

8. A polypeptide as in Claim 5 coupled via said 8th, 80th, 81st, 132nd or 134th amino acid residue, to an enzymatic moiety.

9. A polypeptide as in Claim 5 coupled via said 8th, 80th, 81st, 132nd or 134th amino acid residue, to a toxin.

10. A polypeptide as in Claim 5 coupled via said 8th, 80th, 81st, 132nd or 134th amino acid residue, to a drug.

11. A polypeptide as in Claim 5 coupled via said 8th, 80th, 81st, 132nd or 134th amino acid residue, to an antibody or mitogen.

12. An antibody specifically binding the polypeptide product of Claim 1 but which does not cross react with naturally occurring human IL-2.

13. An antibody as in Claim 12 which is tagged.

14. A manufactured gene capable of directing the synthesis in a selected host cell of the polypeptide of Claim 1.

15. A manufactured gene capable of directing the synthesis in a selected host cell of the polypeptide of Claim 2.

16. A manufactured gene according to Claim 14 wherein the base sequence includes one or more codons, selected from among alternative codons specifying the same amino acid, on the basis of preferential expression characteristics of the codon in a projected host cell.

17. A manufactured gene according to Claim 14 wherein the base sequence includes one or more codons, selected from among alternative codons specifying the same amino acid, on the basis of preferential expression characteristics of the codon in E. coli.

18. A manufactured gene according to Claim 14 wherein base codons specifying the polypeptide include initial and/or terminal codons respectively specifying additional initial and/or terminal amino acids in the polypeptide synthesized.

19. A manufactured gene according to Claim 18 wherein said initial codons specifying additional initial amino acids are codons specifying an initial methionine residue.

20. A manufactured gene according to Claim 14 wherein the base codons specifying the polypeptide are preceded and/or followed by and/or include a sequence of bases comprising a portion of a base sequence which provides a recognition site for restriction endonuclease enzyme cleavage.

21. A manufactured gene according to Claim 14 wherein the base codons specifying the polypeptide are preceded by a sequence of bases comprising a portion of a base sequence which provides a site for ribosome binding.

22. A manufactured gene according to Claim 21 wherein said ribosome binding site is specified by the sequence 5'-CAA GGA GGT-3'.

23. A manufactured gene according to Claim 14 which is labelled.

24. A biologically functional DNA transformation vector including the manufactured gene of Claims 14 or 15.

25. A transformed cell with a vector including a manufactured gene of Claims 14 or 15.

26. A method of removing pyrogens from an IL-2 solution containing pyrogens comprising the steps of:

(a) adjusting the pH of a detergent free IL-2 solution to a pH so that the pyrogens form aggregates of molecular weight greater than the molecular weight of monomeric IL-2; and

(b) separating the aggregates from the IL-2 solution by size.

27. A method as in Claim 26 wherein step (b) comprises repeatedly i) diluting the IL-2 solution and ii) separating the aggregates from the IL-2 solution.

28. A method as in Claim 26 wherein, step (b) comprises separating the aggregates from the IL-2 solution by ultrafiltration or size exclusion chromatography.

29. An IL-2 analog which is the product of the expression in a host cell of a manufactured gene, said analog having one or more of the biological properties of naturally occurring IL-2, wherein at least two original amino acids in helix A and/or helix F have been replaced by substitution amino acids, said substitution amino acids altering the amphiphilicity of each helix containing a substitution amino acid.

30. An IL-2 analog as in Claim 29 wherein the substitution amino acids change the ratio in each helix containing a substitution amino acid, of amino acids having hydrophobic side chains to amino acids having hydrophilic side chains.

31. An IL-2 analog as in Claim 29 wherein said at least two original amino acids are amino acids having hydrophobic side chains and said substitution amino acids are amino acids having hydrophilic side chains.

32. An IL-2 analog as in Claim 29 wherein each of said substitution amino acids has a greater preference for an alpha-helical structure than the original amino acid it replaces.

33. An IL-2 analog which is the product of the expression in a host cell of a manufactured gene, said analog having one or more of the biological properties of naturally occurring IL-2, wherein at least one original amino acid in helix E, and/or at least two original amino acids in helix A, B, and/or B' have been replaced by substitution amino acids, each of said substitution amino acids having a different charge than the original amino acid it replaces.

34. An IL-2 analog as in Claim 33 wherein said original amino acids are hydrophilic amino acids.

35. An IL-2 analog as in Claim 33 wherein said original amino acids are charged amino acids.

36. IL-2 analog as in Claim 33 wherein each of said substitution amino acids has a greater preference for an alpha-helical structure than the original amino acid it replaces.

37. An IL-2 analog which is the product of the expression in a host cell of a manufactured gene, said analog having one or more of the biological properties of naturally occurring IL-2, wherein at least one original amino acid in helix C, D and/or E, and/or at least two original amino acids in helix A, B, B', and/or F have been replaced by substitution amino acids, each of said substitution amino acids having a greater preference for an alpha-helical structure than the original amino acid it replaces.

38. An IL-2 analog as in Claim 37 wherein said original amino acids in helix A, B, B', E and/or F have been replaced by said substitution amino acids, each of said substitution amino acids having a greater

preference for an alpha-helical structure than the original amino acid it replaces.

39. An IL-2 analog as in Claim 37 wherein said at least one amino acid in helix F has been replaced by said replacement amino acid which has a greater preference for an alpha-helical structure.

40. An IL-2 analog as in Claim 37 wherein said substitution amino acids are selected from the group consisting of: Glu, Met, Ala, Leu, Lys, Phe, Gln, Trp, Ile, Val, Asp, and His.

41. An IL-2 analog which is the product of the expression in a host cell of a manufactured gene, said analog having one or more of the biological properties of naturally occurring IL-2, wherein at least one original amino acid in helix A, B', C, D, and/or E, and/or at least two original amino acids in helix B and/or F have been replaced by substitution amino acids, each of said substitution amino acids having a greater preference for an alpha-helical structure than the original amino acid it replaces, and each of said substitution amino acids altering the amphiphilicity of each helix containing a substitution amino acid.

42. An IL-2 analog as in Claim 41 wherein if the original amino acid is hydrophilic, the substitution amino acid is selected from the group consisting of: Met, Leu, Phe, Trp, Ile, and Val; and if the original amino acid is hydrophobic the substitution amino acid is selected from the group consisting of: Glu, Ala, Lys, Gln, Asp, and His.

43. A peptide which is the product of the expression in a host cell of a manufactured gene, said peptide being capable of binding the IL-2 receptor and including at least one of helices A, B, B', and E, and not including helices C, D, and F.

44. A peptide as in Claim 43 wherein at least one original amino acid in helices A, B, B' and E is replaced by a substitution amino acid which has greater preference for an alpha-helical structure than the original amino acid it replaces.

45. A pharmaceutical composition comprising an effective amount of a polypeptide according to Claim 1 and a pharmaceutically acceptable diluent, adjuvant or carrier.

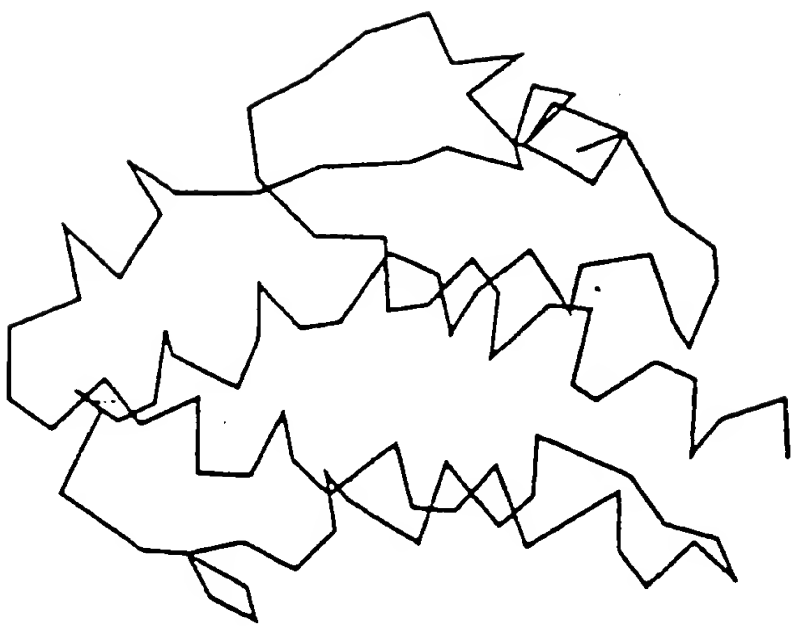
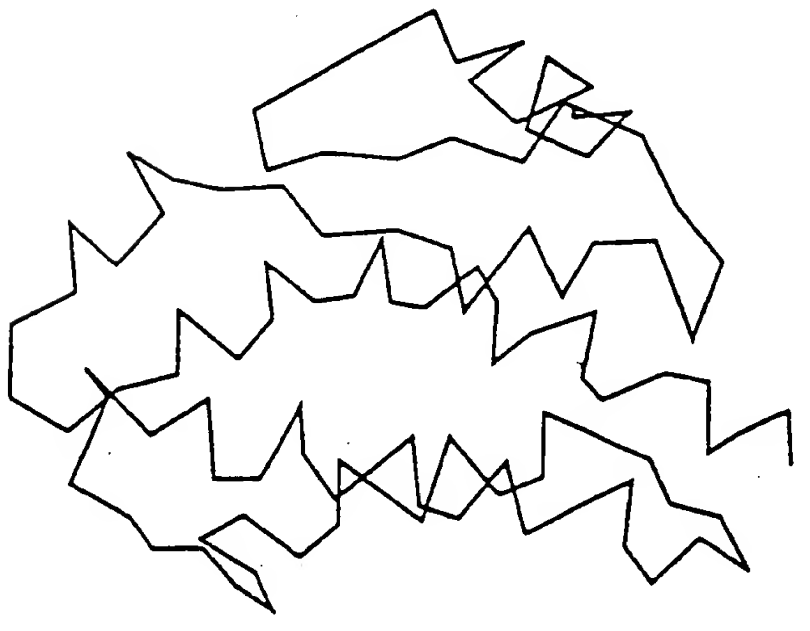


FIG 1-A



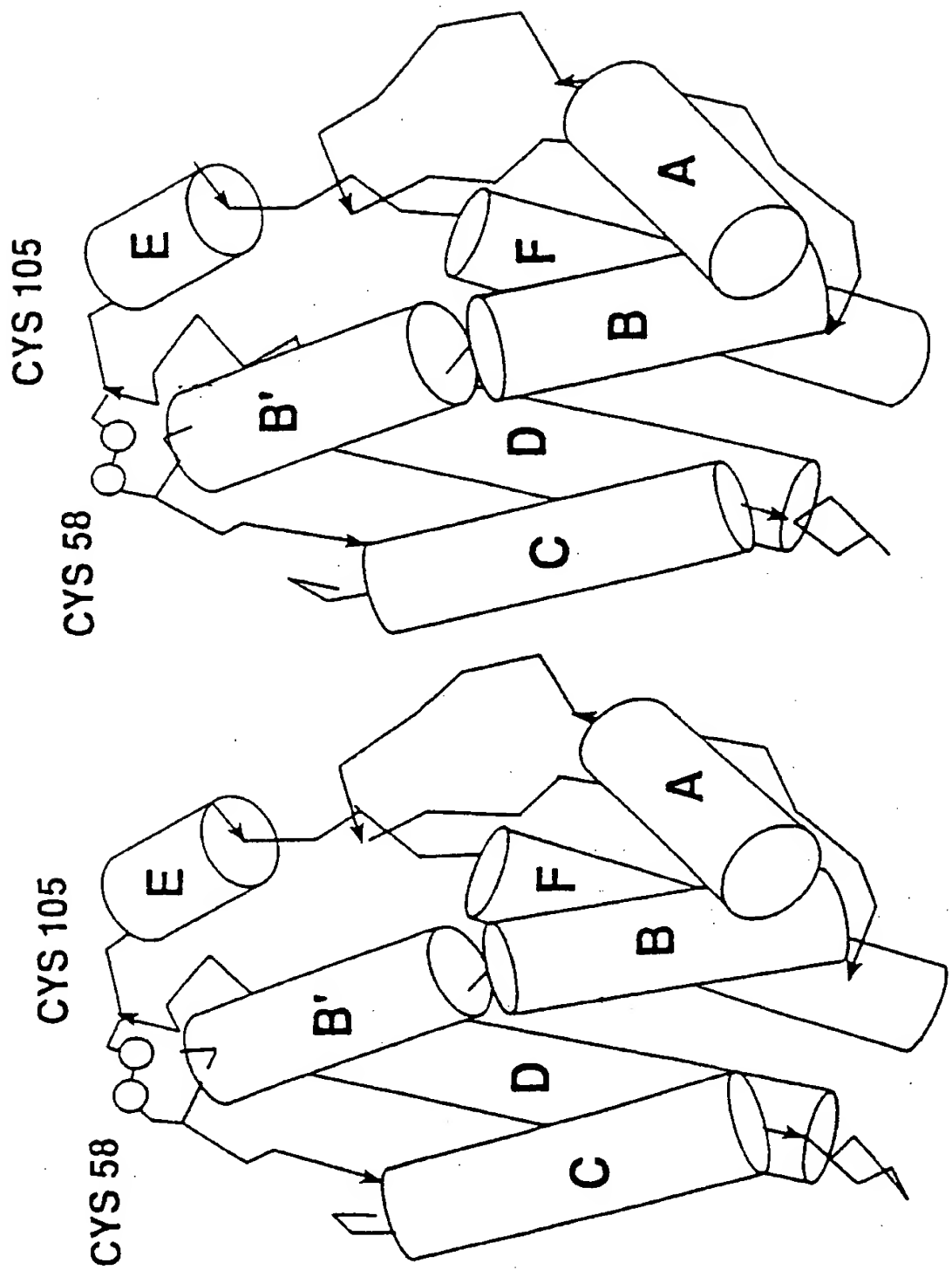


FIG 1-B

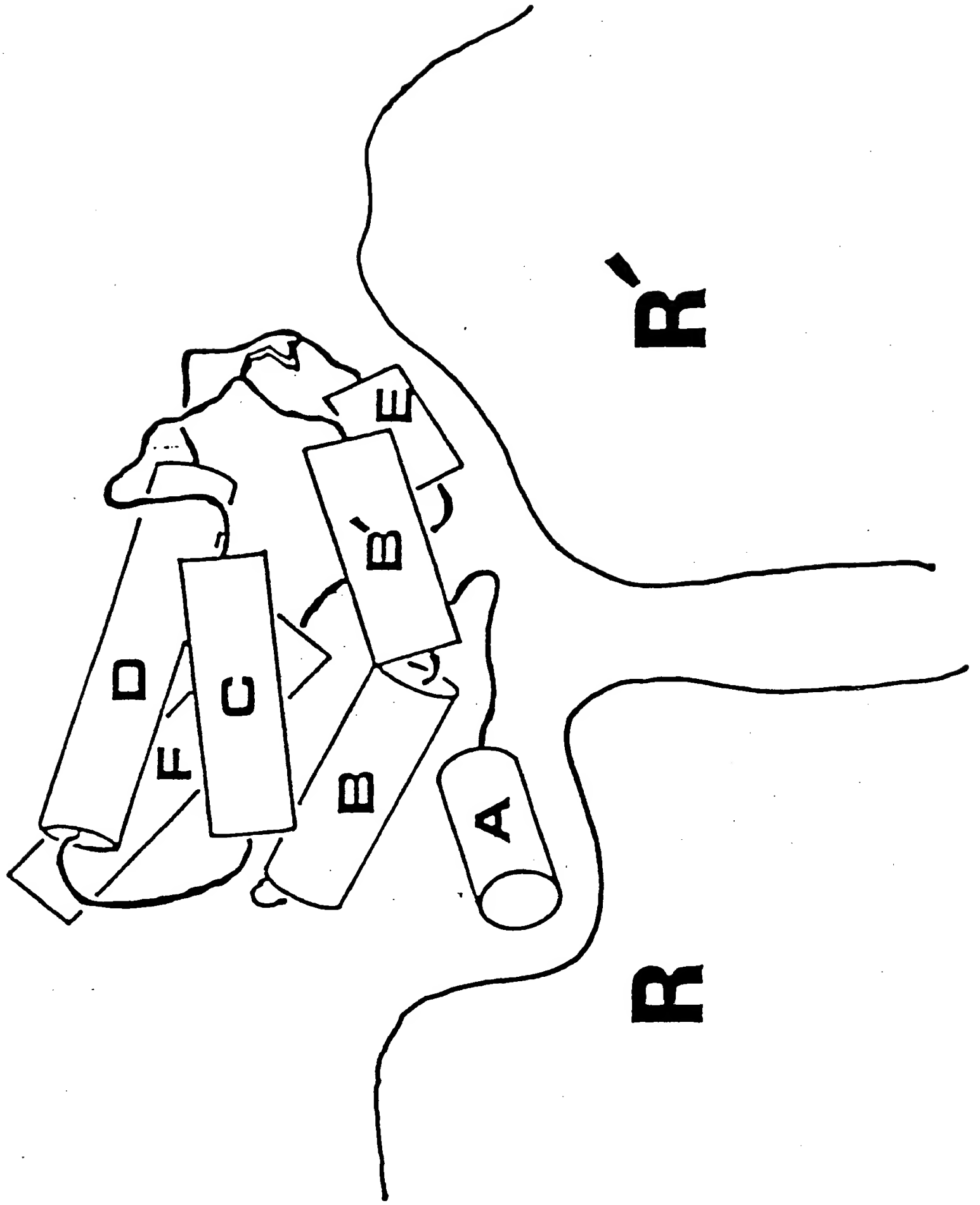


FIG 2

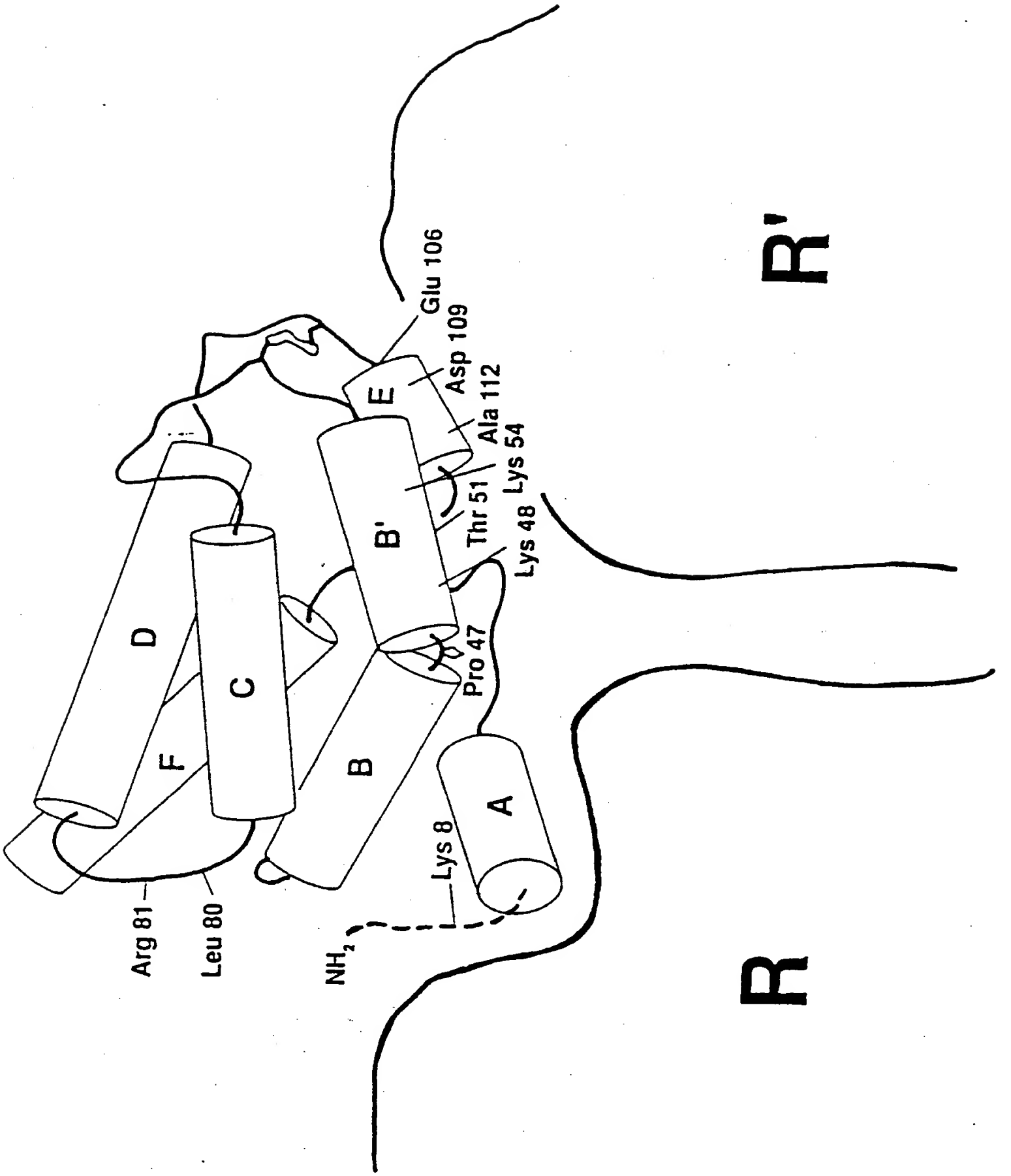

R'
Glu 106
Asp 109
Ala 112
Lys 54
Thr 51
Lys 48
Pro 47
Lys 8
Arg 81
Leu 80
NH_2
A
B
C
D
E
F
B'
R

FIG 3

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/02917

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL.4 C07K 13/00, 17/00, 3/26; A61K 45/00; C12N 15/00; C07H 17/00, 21/04
US CL. 530/351, 387, 402, 412, 417; 435/68, 172.3, 320; 536/27; 424/85.2;

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
U.S.	530/351,387,402,404,405,406,409,412,414,417;435/68,70,172.1 172.3,320;536/27;424/85.2,85.1,85.91,1.1;514/2,8;935/10,11,22

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

Computer search on CAS, Dialog and PIR. For: IL-2 muteins, analogs, derivatives; DNA, antibodies, purification; and sequence search.

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
X / Y	Journal of Biological Chemistry, Vol. 262, Issued 25 April 1987, :Structure-Function Analysis of Human Interleukin-2," (Ju), pages5723-31, See all.	1-6,12-25, 29-45 / 7-11
Y	Science, Vol.234, Issued 17 October 1986, "Structure Activity Studies of Interleukin-2," (Cohen), pages 349-351, See page 349,351.	1-25,29-45
Y	Science, Vol. 238, Issued 13 December 1987,"Three Dimensional Structure of Interleukin-2," (Brandhuber), pages 1707-09, See all.	1-45
X / Y	PCT, A, 85/00817, (Souza), 28 February 1985, See pages 3-4,27.	1-25, 29-45 / 26-28
X / Y	EP, A, 0,200,280, (Koths), 12 October 1986, See pages 6-8,12 and claims.	1-6,14-25, 29-45 / 7-13,26-28
X / Y	PCT, A, 87/04714, (Nishida), 13 August 1987, See abstract.	1-6,14-25, 29-45 / 7-13,26-28

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

20 September 1989

Date of Mailing of this International Search Report

18 OCT 1989

International Searching Authority

ISA/US

Signature of Authorized Officer

GARNETTE D. DRAPER

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
P, X/Y	US, A, 4,761,375, (Clark), 2 August 1988, See claims.	4,30-45, 12-13
Y	US, A, 4,675,382, (Murphy), 23 June 1987, See Cols. 3-4.	9
Y	US, A, 4,636,463, (Altman), 13 January 1987, See cols. 3,5,7 and claims.	1-7,11-13, 29-45
Y	US, A, 4,568,640, (Rubin), 4 February 1986, See claims.	1-6,29-45
Y	EP, A, 0,212,914, (Deeley), 3 April 1987, See abstract and claims.	1-6,14-25, 29-45
Y	Gene, vol. 34, Issued 1985,"Cassette Mutagenesis: An Efficient Method for Generation of Multiple Mutations at Defined Sites," (Wells), pages 315-23, See page 315.	1-6,14-25, 29-45
Y	J. Immunological Methods, Vol. 61, Issued 1983, "Removal of Gram-Negative Endotoxin from Solutions by Affinity Chromatography,"(Issekutz), pages 275-81, See page 279.	26-28
T,P	Nature, Vol. 339, Issued 18 May 1989,"Structural Plasticity Broadens the Specificity of an Engineered Protease,"(Bone), pages 191-195.	1-25, 29-45
T,P	Chemical & Engineering News, Issued 10 April 1989, "Deciphering the Rules of Protein folding," (King), pages 32-54.	1-25 29-45

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows: Group I, claims 1-6,29-45 to IL-2 analogs. Group II, claims 7-11 to IL-2 analog conjugates. Group III, claims, 12-13 to IL-2 analog antibodies. Group IV, claims 14-25 to genes and vectors. Group V, claims 26-28 to a method of removing pyrogen from IL-2. (See Attachments)

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
BLOcp226/79P		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IB99/01424	16/07/1999	16/07/1998
International Patent Classification (IPC) or national classification and IPC		
C07K16/24		
Applicant		
INSTITUT PASTEUR et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
02/02/2000	05.09.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Weijland, A Telephone No. +49 89 2399 7490

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IB99/01424

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-32 as originally filed

Claims, No.:

1-25 as originally filed

Drawings, sheets:

1/16-16/16 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement und r Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	6, 7, 11, 12, 14, 15, 18-25
	No:	Claims	1-5, 8-10, 13, 16, 17
Inventive step (IS)	Yes:	Claims	6, 7
	No:	Claims	1-5, 8-25
Industrial applicability (IA)	Yes:	Claims	1-25
	No:	Claims	

2. Citations and explanations

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Reference is made to the following documents:

D1: ECKENBERG R ET AL., : 'Analysis of human IL-2/IL-2 receptor beta chain interactions: Monoclonal antibody H2-8 and new IL-2 mutants define the critical role of alpha helix-A of IL-2.' CYTOKINE , vol. 9 (7), 1997, page 488- 498

D2: WO 91 02000 A (SERAGEN INC) 21 February 1991 (1991-02-21)

D3: WO 90 00565 A (AMGEN INC) 25 January 1990 (1990-01-25)

D4: MOREAU J-L ET AL., : 'Characterization of a monoclonal antibody directed against the NH2 terminal area of interleukin-2 (IL-2) and inhibiting specifically the binding of IL-2 to IL-2 receptor beta chain (IL-2R-beta)' MOLECULAR IMMUNOLOGY, vol. 32 (14-15), 1995, page 1047-1056

SECTION V

1. Novelty (Article 33(2) PCT)

1.1 The subject matter of claims 1-5, 8-10, 13, 16, 17 is not novel.

Claims 1 and 16, relating to an antibody which binds to a peptide consisting of sequence SEQ ID NO.:2 or 4 and a peptide consisting of the sequence SEQ ID NO.:2 or 4 respectively, are anticipated by D1. The same applies to claims 2, 4 and 17. D1 (abstract; page 489, left column, fifth paragraph) describes an anti human IL-2 mAB (H2-8), produced after immunization with peptide having amino acids 1-30 (SEQ ID NO.: 4, claims 1, 2, 4,16,17) of IL-2, which recognizes the region occupied by Asp20. mAB H2-8 specifically inhibits the IL-2 proliferation of TS1[SPEC0803]. The peptide 1-30 of IL-2 was able to inhibit the binding of mAB H2-8 to IL-2, this peptide adopts a structural conformation close to native IL-2.

Claims 9, 13 and 16, relating to the use of a peptide comprising sequences SEQ ID NO's: 2 or 4 (claims 9, 13) and the peptide having the sequence SEQ ID NO.:2 or 4 (claim 16), are anticipated by D2 and D3. The same applies to claim 17. D2 (page 1, second paragraph) describes the IL-2/diphtheria toxin hybrid (peptide **having** sequence SEQ ID NO.:2 or 4, claim 16, claim 17) shown to inhibit the rejection of transplanted organs and to be a potential therapeutic agent (claims 9 and 13) in the treatment of certain cancers and autoimmune diseases in which IL-

2R plays a role. D3 (page 1, fourth paragraph; page 7, second paragraph) describes that IL-2 (claims 16 and 17) has application in the treatment of neoplastic and immunodeficiency diseases. Pharmaceutical compositions for Il-2 therapy comprising IL-2 and suitable diluents or adjuvants are described (claims 9 and 13).

Claims 1 and 16 are anticipated by D4. The same applies to claims 2, 3 and 17. D4 (abstract; page 1051, left column, paragraph 8) describes mAB 19B11/B and polyclonal antibodies (claims 1-3) recognizing peptide 1-30 (claims 16 and 17) of IL-2 with high affinity.

Claims 5 and 10, relating to a DNA sequence encoding a peptide consisting of sequence SEQ ID NO.:2 or 4 (claim 5) or a vector containing this DNA sequence, are implicitly anticipated by the peptide 1-30 of IL-2 in D1, since this peptide is the translational product after expression of these DNA sequences.

Claim 8, related to a method of inhibiting the activity of an IL-2R, is anticipated by D1. D1 (Figure 5; page 491, left column) describes a method to inhibit the proliferation of the TS1β cell line. Different concentrations of mAB H2-8 were used to reduce the IL-2 proliferation of TS1-β.

1.2 The subject matter of claims 6-7, 11-12, 14, 15, 18-25 is novel.

The subject matter of claims 6-7,11 is not disclosed in the prior art documents. The same applies to claims 12; 14; 15; 18-25.

2. Inventive Step (Article 33(3) PCT).

2.1 The subject matter of claim 11 does not appear to involve an inventive step.

D2 is considered to be the closest prior art. D2 (page 1, second paragraph) describes the use of the IL-2/diphtheria toxin hybrid as potential therapeutic agent in the treatment of certain cancers and autoimmune diseases in which IL-2R plays a role. Claim 11 differs from D2 in that claim 11 describes the use of a vector

containing SEQ ID No's 2 or 4 for the preparation of a medicament useful to induce in a patient selected useful activities of IL-2.

The technical problem to be solved would appear to reside in finding an alternative molecule useful to induce in a patient IL-2 activity.

The skilled person, equipped with the knowledge of D2, would be motivated to turn to D1 for the solution of this particular problem. This document is concerned with a similar problem, that is the search for functional homologs of IL-2. It is there suggested to solve the problem by using a mutant peptide comprising the 1-30 amino acids of IL-2. This suggestion essentially corresponds to the feature which distinguish claim 11 from the prior art, since the vector mentioned in claim 11 expresses SEQ ID NO.:4. The skilled person would know how to derive the DNA sequence from the peptide encoding amino acids 1-30 of IL-2 for use in the vector of claim 11.

2.2 The subject matter of claim 18-25 does not appear to involve an inventive step.

Dependent claims 18-25 do not contain any features which, in combination with the features of claim 16 to which they refer, meet the requirements of the PCT in respect of inventive step, since the substitution of the peptide sequence of IP130 with the conservative amino acids form merely obvious alternatives for the skilled person without resulting in any special effect whatsoever.

2.3 The subject matter of claim 12, 14 and 15 does not appear to involve an inventive step.

Dependent claims 12, 14 and 15 do not contain any features which, in combination with the features of claim 9 to which they refer, meet the requirements of the PCT in respect of inventive step, since the use of peptides in admixtures comprising a cytokine to increase the activity without resulting in any special effect whatsoever are merely obvious alternatives for the skilled person.

2.4 The subject matter of claim 6-7 would appear to involve an inventive step. D1 is considered to be the closest prior art. D1 (page 489, left column, fifth

paragraph) describes that the peptide having the amino acids 1-30 of IL-2 was able to inhibit the binding of mAB H2-8 to IL-2 and that this peptide adopts a conformation close to native IL-2. Claims 6-7 differ from D1 in that said claims describe:

- a method of detecting in vitro the presence of activity of IL-2R comprising incubation with the 1-30 IL-2 peptide (claim 6).

- methods for inhibiting the activity of an IL-2R by using the 1-30 IL-2 peptide (claim 7) as inhibitor.

These methods are not suggested in the prior art, since it was not shown in the prior art documents that the 1-30 IL-2 peptide can bind to IL-2R, despite that the peptide can inhibit the binding of mAb H2-8 to IL-2.

SECTION VII

3. The term "bind" in claim 8 needs to read probably "binding".

4. Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D2 and D3 is not mentioned in the description, nor are these documents identified therein.

5. The application should be self contained (see further Guidelines C-II 4.17) and phrases "and incorporated by reference..." as mentioned on page 19 (line 12) contravene this requirement.

6. The references made to the Figure 8(A) (page 5, line 15), Figure 8(B) (page 5, line 17), and 8(C) (page 5, line 22) are not clear.

SECTION VIII

7. Claim 4 is not clear (Article 6 PCT). In said claim a hybridoma is identified by way of a trivial designation, which is meaningless to a person skilled in the art.

In order to meet the requirements of Article 5 and Rule 13bis PCT, copies of the deposition receipts or an equivalent proof needs to be present (see the Guidelines C-II 6.3).

8. The vague and imprecise statement in the description on page 32 (second paragraph) implies that the subject-matter for which protection is sought may be different to that defined by the claims, thereby resulting in lack of clarity (Article 6 PCT) when used to interpret them (see also the PCT Guidelines, PCT/GL/3 III, 4.3a).

9. The applicant attributes to the term "R groups" in claims 18-20 a special meaning (see also page 12, line 32 of the description), which was not generally known in the technical field concerned at the relevant filing date and contravenes thereby the requirements of Article 6 PCT.

10. Claims 9 and 13 suffer from lack of clarity (Article 6 PCT), because they are formulated as second medical indication claims, but are not defined by a medical indication. The passage "..useful to induce in a patient selected useful activities.." in claim 9 or "..in an amount able to induce said useful activities" in claim 13, define an effect to be obtained, rather than a medical indication (i.e. a disease).